Covalent immobilisation of β-Galactosidase from *Escherichia coli* to commercially available magnetic nanoparticles for the removal of lactose from milk

by

Chantelle Pretorius

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Supervisors:

Prof. Pieter Swart

Dr. Karl-Heinz Storbeck

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Declaration

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Abstract

β-Galactosidase of *Escherichia coli* is the equivalent of lactase in humans and has the ability to bind and hydrolyse lactose. Lactase deficiency is a common phenomenon present in almost 70 % of the world's population. This has resulted in greater than before demands on the food processing industry to develop a method that will allow for the hydrolysis of the disaccharide lactose in milk but will also allow for the removal of the remaining active enzyme.

In this thesis, a new method, that is bio-specific and well characterized for the removal of lactose from a lactose containing solution, is described. The E537D mutated version of β-Galactosidase, which has a much lower activity compared to the wildtype and is able to bio-specifically bind lactose for longer periods, was covalently immobilised to commercially available magnetic nanoparticles (fluidMAG-Amine) via two coupling strategies. Glutaraldehyde is a cross-linking agent that reacts with amine groups, while N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) is a coupling agent that activates carboxylic groups. These agents are widely used for the coupling of biomolecules to solid supports.

The covalently coupled fluidMAG-E537D β-Galactosidase particles were characterized regarding retained enzymatic activity and ability to bind and physically remove lactose from a lactose containing solution by applying an external magnetic field, after lactose binding, to the enzyme-particle complex in solution.

Each component aimed at yielding this functionally immobilised enzyme complex was studied and optimized to contribute to the development of this novel technique, which is affordable and simple, for the removal of lactose from solution for the ultimate production of lactose free milk.

Results indicated the glutaraldehyde method of β-Gal cross-linking to fluidMAG-Amine to be the preferred strategy since it allowed an increased carrier capacity of protein to the particles. The glutaraldehyde cross-linked protein also exhibited a two-fold higher activity than the EDC coupled protein. Furthermore, the glutaraldehyde cross-linked fluidMAG-E537D β-Gal was able to physically remove 34 % of the lactose from a 0.2 nmol/L lactose in solution. This, therefore, confirmed the potential use of this novel technique in the food processing industry.

Opsomming

β-Galaktosidase vanaf *Escherichia coli* is dieselfde as laktase in mense en beskik oor die vermoë om laktose te bind en te hidroliseer. 'n Gebrek aan laktase kom algemeen voor en ongeveer 70 % van die wêreldbevolking ly hieraan. Laasgenoemde het daartoe gelei dat daar meer druk as vantevore op die voedselproduksie industrie is om 'n metode te ontwikkel waarmee die hidrolise van die disakkaried laktose in melk moontlik sal wees asook die verwydering van die oorblywende aktiewe ensiem.

In hierdie tesis word 'n nuwe metode beskryf wat biospesifiek en goed gekarakteriseer is vir die verwydering van laktose vanuit 'n laktose bevattende oplossing. Die E537D gemuteerde weergawe van β-Galaktosidase, wat beskik oor 'n baie laer aktiwiteit as die wildetipe asook die vermoë om laktose biospesifiek vir langer periodes te bind, is kovalent geïmmobiliseer op kommersieel beskikbare magnetiese nanopartikels (fluidMAG-Amine) via twee koppelingsstrategieë. Glutaraldehied is 'n kruisbindingsagent wat met amino groepe reageer, terwyl EDC 'n koppelingsagent is wat karboksie groepe aktiveer. Hierdie agente word algemeen gebruik vir die binding van biomolekules aan soliede matrikse.

Die kovalent gekoppelde fluidMAG-E537D β-Galaktosidase partikels is gekarakteriseer met betrekking tot behoue ensimatiese aktiwiteit en vermoë om laktose te bind en fisies te verwyder vanuit 'n oplossing wat laktose bevat deur 'n eksterne magneetveld op die ensiem-partikel kompleks in oplossing toe te pas, nadat die binding van laktose plaasgevind het.

Elke komponent van hierdie funksioneel geïmmobiliseerde ensiemkomplekse is ondersoek en geoptimaliseer met die doel om by te dra tot die ontwikkeling van 'n nuwe tegniek wat bekostigbaar en eenvoudig is vir die verwydering van laktose vanuit 'n oplossing vir die uiteindelike gebruik in die produksie van laktose-vrye melk.

Resultate het getoon dat die glutaraldehied metode van β-Gal kruisbinding op fluidMAG-Amine verkies word aangesien dit 'n verhoogde draerkapasiteit van proteïene op die partikels moontlik maak. Die glutaraldehied gekoppelde proteïene beskik ook oor twee keer meer aktiwiteit as die EDC gekoppelde proteïene. Die glutaraldehied gekoppelde fluidMAG-E537D β-Gal kon 34 % van die laktose teenwoordig in 'n 0.2 nmol/L laktose oplossing fisies verwyder. Hierdie het dus die potensiële gebruik van hierdie nuwe metode in die voedselproduksie industrie bevestig.

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(06-11-1930 – 18-02-2012)

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List of Abbreviations

In order of appearrance

BC	Before Christ
β-Gal	β-Galactosidase
MNP	Magnetic Nanoparticles
E	Glutamic acid
D	Aspartic acid
E. coli	*Escherichia coli*
His_6	Hexahistidine
IMAC	Immobilized Metal-chelate Affinity Chromatography
YASARA	Yet Another Scientific Artificial Reality Application
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
BCA	Bicinchoninic acid
ONPG	o-nitrophenol-β-D-galactoside
HPLC	High Performance Liquid Chromatography
GOS	Galacto-oligosaccharides
UDP	Uracil-diphosphate
LCT	Lactase-phlorizin hydrolase
HL	Hypolactasia
Glu	Glutamic acid
Met	Methionine
His	Histidine
FBR	Fluidised Bed Reactor
PBR	Packed Bed Reactor
MR	Membrane Reactor
SC	Starter Cultures
LB	Luria-Bertani
IPTG	Isopropyl-1-thio-B-d-galactopyranoside
PMSF	Phenylmethanesulfonylphosphonate
SDS	Sodium Dodecyl Sulphate
PAGE	Polyacrylamide Gel Electrophoresis
BSA	Bovine Serum Albumin
TEMED	N,N,N'N'-tetramethilene diamine
TBST	Tris Buffered Saline Tween
AP	Alkaline Phosphatase
HRP	Horseradish peroxidase
PBS	Phosphate Buffered Saline
PVL	Portable Vector Language

pI	Isoelectric point
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared vibrational spectroscopy
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
SEM	Standard error of the mean
GC	Gas Chromatography
TLC	Thin Layer Chromatography
MS	Mass Spectrometry
RI	Refractive index
PAD	Pulsed amperometric detector
CPM	Counts per million
GRAS	Generally Recognized as Safe
GMP	Good Manufacturing Practice

Chapter 1

INTRODUCTION

1.1 Background

There is a growing demand for lactose free/reduced products in the world today. This is due to the majority of the world's population (70 %) being unable to digest significant amounts of lactose (the disaccharide sugar in milk).

Lactose intolerance is caused by inadequate amounts of lactase, which is an enzyme responsible for the digestion of lactose, being expressed in the small intestinal villi. Interestingly the degree of lactase deficiency differs between patients. It has been shown that there are three main types of lactase deficiency: primary, secondary and congenital [1]. Gastrointestinal symptoms are the most common effect of the malabsorption of dietary lactose in the small intestine due to the lack of or insufficient lactase expression [1].

The most common type of lactose intolerance, primary adult type, varies significantly between individuals from different areas/regions as well as between different ethnic groups [2; 3]. According to Swagerty *et al.* [1], it is present in 15 percent of Northern Europeans, 80 percent of Blacks and Latinos and up to 100 percent of Asians and American Indians. Simoons [2] hypothesized that this phenomenon is due to historical milking habits and genetic selection. Research has indicated that populations from areas (Europe

and Africa) that milked dairy animals (cows, goats, sheep and water buffalo) and consumed these dairy products in the period roughly from 4000 to 1200 BC have low incidents of adult lactose intolerance [2]. In contrast to this, high incidences of lactose intolerance occur in adults in traditionally non-milking areas i.e. the Pacific, East and Southeast Asia and the New World [2]. It therefore seems likely that the global variation in lactose intolerance may have resulted from a genetic basis for primary adult lactose intolerance accompanied by a form of selection for tolerance due to milking habits to allow for the consumption of lactose-rich dairy products.

1.2 Current practice in the production of lactose free products

Currently, some lactose free/reduced products are on the market. The manufacturers of these products (example EasyGest, Parmalat SA (Pty) Ltd.) generate these by adding the free lactase enzyme to the milk. This enzyme breaks down the lactose into glucose and galactose (monosaccharide sugars), which the body can absorb. Some lactose free milk producing companies include several ultra-centrifugation steps after the complete hydrolysis of the lactose present. This step removes some of the excess monosaccharide sugars. Unfortunately, due to the higher relative sweetness of glucose and galactose in comparison to lactose, the resulting product is very sweet. A consumer study revealed that this very sweet taste is perceived as negative. This aspect will be discussed in greater detail in Chapters 2 and 3.

1.3 Focus of this study

The central idea at the start of this study was to develop a novel technique for the removal of lactose from milk to contribute to, and boost, the food

processing industry with regards to the production of lactose free/reduced dairy products.

Lactose, the sugar for which the intolerance is named, is converted to glucose and galactose via enzymatic hydrolysis. This creates a new problem due to the higher relative sweetness of the combination of glucose and galactose as compared to lactose.

This novel method would include the removal of a portion of the lactose from milk while minimising the hydrolysis to glucose and galactose. It is important, however, that some of the lactose is hydrolyzed to the monosaccharide sugars in order to account for the loss in sugar caused by the removal of lactose.

To accomplish lactose binding but minimise lactose hydrolysis, a E537D mutated form of β-Galactosidase (β-Gal) will be used that exhibits much lower hydrolysis activity compared to the wildtype β-Gal, but similar substrate binding capabilities. For the removal of lactose from milk, the E537D-β-Gal would be immobilised to a solid support (magnetic nanoparticles (MNPs)), added to the milk and allowed to bind some of the lactose present. This is possible since the E537D mutated form of the enzyme allows for the formation of a stable enzyme-substrate complex with an extended half-life. The MNPs, protein coated with the lactose bound in the active site, would subsequently be manipulated to the side of the milk container through an external magnetic field and the milk with the reduced lactose content decanted.

1.3.1 Research question

The ultimate research aim was to develop a new sustainable technique for the removal of lactose from milk that was also 1) affordable, 2) simple, 3) more effective than the available techniques and 4) suitable for industrial application.

1.3.2 Previous research on this project by our group

Amanda Dodd [4], a M.Sc Biochemistry student of Prof. Pieter Swart in our laboratory at the department of Biochemistry, Stellenbosch University, started with this project in 2009. She performed the cloning and site-directed mutagenesis of β-Gal from *Escherichia coli* (*E. coli*) to produce a hexahistidine (His_6)-tagged E537D mutant of the enzyme of interest. After mutagenesis, the wildtype and mutant β-Gal were kinetically characterized. The calculated kinetic parameters for the wildtype β-Gal was a K_m value of 0.22 mM and a V_{max} of 230 μmol/min/mg protein, for the E537D β-Gal these values were 0.27 mM and 2 μmol/min/mg protein, respectively [4]. The kinetic characterization of E537D β-Gal indicated that the E537D mutation ensured the binding of the substrate (lactose), but limited the hydrolysis to glucose and galactose as well as the conversion to allolactose, which is an isomer of lactose and the natural inducer of lac operon in *E. coli* [4].

Dodd went further and immobilised the wildtype and E537D β-Gal mutant to Dynabeads (Invitrogen Dynal AS, Oslo, Norway, Cat. no. 101.03D) through Immobilised Metal-Chelate Affinity Chromatography (IMAC) by non-covalent interactions between Co^{2+} on the nanobead surface and the His_6-tag cloned on the N-terminal of the protein/enzyme. The immobilised wildtype and E537D β-Gal enzymes (45 μg protein per mg Dynabeads) were also characterized with respect to their ability to bind, hydrolyze and extract lactose from a lactose containing solution. Results obtained indicated that the mutant immobilised through IMAC to Dynabeads was able to bind and remove approximately 15 % of 0.2 nmol lactose [4].

This study incorporated directed, IMAC, non-covalent interactions for the coupling of β-Gal to MNPs. Directed coupling has advantages over random strategies since the enzyme is orientated on the MNPs, which might improve the accessibility of the active site to lactose. On the other hand, IMAC non-covalent immobilisation is not ideal for implementation in industry due to the

possibility of metal and enzyme leakage into the milk.

It is to prevent these possible leakages that this study incorporates covalent coupling of β-Gal to MNPs. To accomplish covalent coupling, the particles must be functionalized accordingly.

1.3.3 Goal of this study

Covalently immobilise purified E537D β-Gal through random immobilisation strategies to commercially available MNPs and to subsequently test the ligand binding ability of the formed complexes as well as the ability to remove lactose from a lactose containing solution.

1.3.4 Objectives

To accomplish this goal, several objectives needed to be met, these were:

1. Verification and characterization of the cloned wildtype and E537D mutated β-Gal enzyme.
2. Identification of accessible functional surface groups to be targeted during covalent coupling to MNPs.
3. Covalent immobilisation of E537D β-Gal to commercially available MNPs.
4. Characterization of MNP-immobilised E537D β-Gal.
5. Upscale evaluation.

1.4 Experimental tasks

To ensure experimental progress and for documenting purposes the study was divided into several experimental aims and tasks.

1.4.1 Project controls: Characterization of Wildtype and E537D β-Gal (Chapter 3)

- Aim 1: Characterization of β-Gal by repeating previous work done by Dodd [4] to determine the reproducibility of results and to compare methodologies.

The work described in the first experimental chapter was necessary to repeat the expression of the cloned and mutated β-Gal enzyme as well as to purify the enzyme through IMAC in order to obtain a pure sample for the subsequent characterization and immobilisation purposes. Sufficient controls were included during all the steps to verify the presence of β-Gal and to eliminate contamination by other proteins. The kinetic parameters (K_m and V_{max}) of the pure wildtype and mutant β-Gal were also determined in order to compare a microtitre plate method [5] to the test-tube method previously used by Dodd [4], as well as to verify and compare the activity of the pure wildtype and mutant β-Gal.

1.4.2 β-Gal structure investigation (Chapter 4)

- Aim 2: Investigation and identification of the functional surface groups of β-Gal accessible for covalent coupling to MNPs.

After the experimental characterization of β-Gal it was necessary to investigate the three dimensional structure and surface topography of the protein to determine the optimum functional group to be targeted during random covalent immobilisation to MNPs. Two well-known molecular visualization tools (DS ViewerPro^{TM} Version 5.0 and Yet Another Scientific Artificial Reality Application (YASARA)) were used to calculate and visualize the basic and acidic amino acid content of β-Gal. This provided useful information in deciding which of the functional groups should be targeted during the subsequent covalent coupling step to limit enzyme structural changes and activity loss.

1.4.3 A comparison between EDC and Glutaraldehyde for random covalent immobilisation (Optimized) of E537D β-Gal to commercially available surface activated MNPs (fluidMAG-Amine) (Chapter 5)

- Aim 3: Covalent immobilisation of β-Gal to MNPs as well as a comparison between the immobilisation potential of two different covalent attachment strategies. The results would indicate which of the immobilisation strategies employed were optimal regarding carrier capacity.

The three dimensional structure investigation revealed the optimum functional surface groups to be targeted. This allowed the selection of the appropriate commercially available MNPs as well as cross-linking agents to accomplish covalent immobilisation. E537D β-Gal was covalently immobilised randomly to commercially available surface activated MNPs according to two immobilisation strategies i.e. a coupling agent (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)) and a cross-linking agent (glutaraldehyde). The amount of protein (mg), temperature (^{o}C) and absorption time (min) were optimized for the two immobilisation strategies. The amount of protein immobilised was estimated according to manufacturers instructions for the Pierce bicinchoninic acid (BCA) protein assay kit that allowed for the "colorimetric detection and quantification" of the total protein present in the supernatant before and after immobilisation [6]. The covalent attachment was also verified through several techniques.

1.4.4 Covalently immobilised E537D β-Gal activity optimization and characterization (Chapter 6)

- Aim 4: Investigation and calculation of the retained activity and ligand binding ability of E537D β-Gal after random, covalent immobilisation

to MNPs through either EDC or glutaraldehyde cross-linking. This step would indicate which of the random covalent coupling strategies of β-Gal to MNPs was optimum in yielding a functionally immobilised enzyme.

The activity of the immobilised E537D β-Gal was investigated through β-Gal enzymatic activity assays, with the use of o-nitrophenol-β-D-galactoside (ONPG) as substrate [5]. The ligand binding ability of immobilised β-Gal was investigated through radioactive binding studies using radioactive lactose, otherwise referred to as D-glucose-1-^{14}C, since it is only the glucose moiety of the lactose that is labelled. The feasibility of the technique for possible industrial application was tested by exposing the MNPs, with surface immobilised E537D β-Gal, to a radiolabeled lactose solution and the subsequent quantification of the D-glucose-1-^{14}C left in the solution after lactose extraction using radioactive partition High Performance Liquid Chromatography (HPLC).

1.4.5 Upscale evaluation (Chapter 7)

Calculations regarding the possible upscaling of this novel technique for the implementation thereof in the food processing industry was conducted and evaluated.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

The down-regulation of expression of the lactase enzyme in the small intestinal villi of humans results in a common deficiency known as lactose intolerant. This lactase found in humans is the iso-enzyme of the β-Gal family of enzymes present in a large variety of organisms that have important applications in a variety of research fields [7; 8]. The main difference between lactase and β-Gal is the size. β-Gal is 464 kDa, while lactase is a mere 150 kDa. Not only does β-Gal allow for the hydrolysis of lactose, but a significant amount of galacto-oligosaccharides (GOS) are also formed during lactose hydrolysis by β-Gal with exceptional nutritional value in prebiotic foods [7]. Over 65 sources of this particular enzyme provide researchers with a broad range of enzymatic properties and structures that could ultimately be investigated individually [9]. β-Gal from *E. coli* is of particular importance to this study due to the well understood nature and simplicity of working with this model organism. Several important topics and techniques related to this study have been investigated by numerous research groups. By referring to these related topics, as well as highlighting the current health issue at hand, a better understanding of the current research in this field as well as a more elaborated proposal of how this

project fits into the bigger scientific world is formulated.

2.2 Lactose intolerance

As previously discussed, lactose intolerance is a well known, common deficiency, which is characterized by the presence of inadequate amounts of the lactase enzyme in the small intestine.

2.2.1 Prevalence

It is estimated that as much as 70 % of the world's population is lactase deficient and have difficulty in consuming milk and dairy products [10]. According to Madry *et al.* [11], lactase deficiency is therefore the world's most common enzyme deficiency in humans.

Some speculation has raised the issue that it is possible for lactase deficiency to be the "natural" or "normal" state due to the wide variation in prevalence (Table 2.1). Lloyd *et al.* [12] is of the opinion that it is possible that an "abnormal" mutation occurred in the Northern European populations allowing selective advantage to groups that consumes dairy products and therefore allowing significant lactase activity well into adult life. However, there are still many controversial views regarding this statement.

Table 2.1 summarizes the prevalence for lactose intolerance among different ethnic backgrounds. It is interesting to note that both American Indians and Asians have prevalence for primary lactase deficiency of nearly 100 %. These groups are identified by Simoons [2] as populations originating from traditionally non-milking cultures who did not consume dairy products and therefore did not develop a tolerance for lactose.

Table 2.1: Prevalence of primary lactase deficiency in various ethnic groups. As summarized by Sahi in 1994 [3].

Group	*Prevalence (%)*
Northern Europeans	2 to 15
American whites	6 to 22
Central Europeans	9 to 23
Indians -Northern subcontinent -Southern subcontinent	 20 to 30 60 to 70
Hispanics	50 to 80
Ashkenazi Jews	60 to 80
Blacks	60 to 80
American Indians	80 to 100
Asians	95 to 100

2.2.2 Lactose biosynthesis

There are three enzymes that are closely related to the biosynthesis of lactose (Figure 2.1) i.e. UDP-glucose pyrophosphorylase, UDP-galactose-4-epimerase and lactose synthetase. UDP-glucose pyrophosphorylase and UDP-galactose-4-epimerase together with phosphoglucomutase are responsible for the conversion of glucose-6-phosphate to UDP-galactose, while glucose-6-phosphatase converts a portion of glucose-6-phosphate to glucose. The synthesis of lactose from these two monosaccharide sugars is catalyzed by lactose synthetase partially regulated by α-lactalbumin (a milk protein).

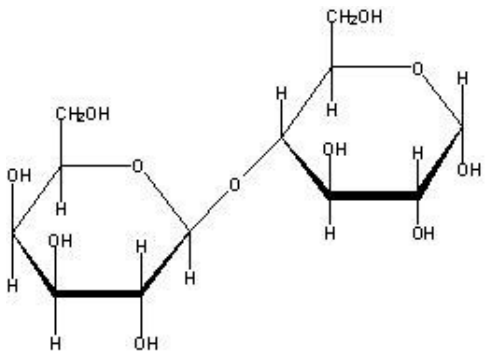


Figure 2.1: Chemical structure of lactose, $C_{12}H_{22}O_{11}$.

This process, through which lactose is synthesized, is confined to female mammals and occurs only in a single tissue, the mammary gland [13]. The enzymatic reaction, through which lactose is synthesized, was first confirmed by Watkins and Hassid [14] and it was said to occur according to the following reaction:

$$UDP - galactose + glucose \longrightarrow lactose + UDP$$

The complexity of the biochemical control mechanisms that appears to be involved in the regulation of lactose biosynthesis was first investigated in detail by Palmitter in 1969. It was experimentally observed by Palmitter [15] that the lactose content of milk varies inversely to the total fat and protein content in milk but no apparent explanation was given for this phenomenon.

To conclude, α-Lactalbumin and the galactosyl transferase, the two components of lactose synthetase, are suggested to play a critical role in the hormonal regulation of control mechanisms in lactose biosynthesis [13].

2.2.3 Lactose content of milk

The fat, fatty acid and protein content of milk can be changed/controlled over a certain range but the lactose content is more or less stable [16]. The only way in which the lactose content can be altered significantly is through extreme dietary manipulations [16]. Not only is lactose the major milk carbohydrate in most species but, according to Squires [16], it is also the most important osmotic component. It is therefore apparent that any changes in milk lactose synthesis would be accompanied by changes in water volume and therefore result in changes in milk yield.

Some animal species, like the kangaroo and bear, have very little lactose in their milk while lactose contributes to up to 7 % of the total milk composition of other species like humans and donkeys.

The lactose content of several dairy products have been investigated by many groups. Although there might be slight differences between the calculated values, the general consent is that reduced fat (1 and 2 %) milk has a higher lactose content (135 mmol/L) when compared to regular fat or raw milk (130 mmol/L), but can vary from 111 - 155 mmol/L with the same variability present in low fat yoghurts [17].

2.2.4 Lactose digestion

Lactase (EC.3.2.1.108), also known as lactase-phlorizin hydrolase (LCT), is an enzyme located in the microvili of the small intestine enterocyte and is responsible for the cleaving/hydrolysis of the dietary disaccharide lactose into the more absorbable monosaccharides, glucose and galactose, for the subsequent transport across the plasma membrane (Figure 2.2) [11].

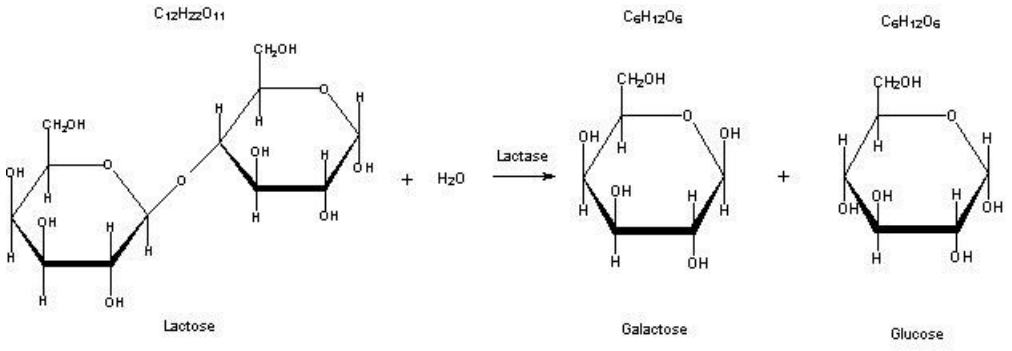


Figure 2.2: Lactase catalyzed lactose digestion. Lactose ($C_{12}H_{22}O_{11}$) is hydrolyzed (H_2O) by lactase to yield Galactose ($C_6H_{12}O_6$) and Glucose ($C_6H_{12}O_6$).

2.2.5 Lactase deficiency

The gastro-intestinal symptoms resulting from lactase deficiency (hypolactasia (HL)) in humans are summarized by Madry *et al.* [11] and Swagerty *et al.* [1] as the result of decreased lactase activity in the small intestinal villi that causes the unabsorbed disaccharide sugar, lactose, to accumulate, which in turn results in the osmotic effect where more fluid is attracted into the bowel

lumen. Furthermore, this fluid influx, caused by the high osmolality of the sugars present, is accompanied by the production of gas through microflora fermentation of the bacteria-affected-unabsorbed-lactose that remains in the colon. In addition, this gas results in the cleaving of lactose and the product, monosaccharide sugars (glucose and galactose) further increase the osmotic pressure in the bowel. Hence the net result of lactose ingestion by lactose deficient individuals is a considerable rise in fluid and gas in the bowel [1; 11].

The three variations in lactase deficiency can be described as primary, secondary and congenital. Primary lactase deficiency refers to the most common phenotype i.e. adult type hypolactasia [11] that occurs after weaning and continues throughout adult life. Here the expression levels of lactase in the small intestine declines over time [1]. This has been hypothesized to be the "normal" or "wildtype" state and that individuals prehistorically developed a tolerance for lactose due to exposure to it [2]. Secondary or acquired lactase deficiency is as a result of any of a range of gastrointestinal illnesses. Lastly, congenital lactase deficiency is rare and is characterized by a complete lifelong absence of lactase [1].

2.2.6 Symptoms and current solutions

Sufferers of lactase deficiency are unable to digest significant amounts of lactose and may develop certain clinical symptoms such as flatulence, blanching, cramps, abdominal pain and distention, and loose, watery stools following the indigestion of lactose containing food products [1; 10]. The only direct measurement of lactase, to diagnose lactase deficient patients, requires a small intestine biopsy; patients usually decline this procedure due to its invasive nature [11]. Other diagnostic tests include the analysis of blood glucose levels or breath hydrogen levels after the ingestion of a predetermined dose of lactose (1 to 1.5 g lactose per kg body weight) [1]. The blood glucose test is directed at measuring the carbohydrate metabolism while the hydrogen breath test is

directed towards measuring the production of hydrogen and other gasses in the colon [1].

Currently the solution to, or treatment of, lactase deficiency is simple dietary adjustments. For some this may be a solution but for those that suffer from congenital lactase deficiency this is not ideal. Apart from lactose, milk and dairy products contain other essential nutrients like calcium. Dairy products provide an astounding 75 % of the calcium that is available through food supply. Without this natural calcium resource, adult patients should maintain a daily calcium intake of 1200 to 1500 mg, which would in this case require the taking of daily supplements [1]. Other temporary treatments include the taking of commercially available lactase enzyme supplements like *Dairy Ease*, *Equate* and *Lacteeze* lactase products.

Some lactose free or lactose reduced dairy products are currently available on the market but these products are produced through the hydrolysis of the lactose present in milk and not the actual removal of the lactose. It follows that these products then have a significantly sweeter taste than normal lactose containing milk products since these lactose free products now contain two moles of sugar (glucose and galactose) instead of only one mole (lactose). Furthermore, the relative sweetness of these sugars, when compared, also indicates that glucose and galactose are both significantly sweeter than lactose [18].

All the current treatments for lactose intolerance as well as commercially available lactose free products involve the lactase enzyme. This can be either directly as dietary supplements or indirectly as part of the manufacturing process for the production of these lactose free products. It would thus be beneficial to this study to investigate this enzyme (β-Gal) and to determine which of its characteristics can be manipulated to contribute to the success of this project.

2.3 β-Galactosidase

β-Gal (EC.3.2.1.23) is the hydrolase enzyme of interest for this study and also known in biochemistry as a retaining glycosidase. This means that the enzyme, β-Gal, catalyzes the conversion of lactose to allolactose as well as the hydrolysis of lactose into glucose and galactose through a double displacement reaction where the product and starting states have the same stereochemistry [19]. β-Gal is dependent on mono- and divalent cations for full activity and the most common cations used for kinetic assays are sodium, potassium, magnesium and manganese [19].

The enzyme can also be used in transglycosylation of lactose to synthesize GOS which is a prebiotic used in many food products [9]. Figure 2.3 is a schematic to explain this function of β-Gal [20]. β-Gal has also been obtained and/or isolated from a wide variety of sources, such as plants, animals and microorganisms, including bacteria, fungi and yeast. It is also general practice to refer to β-Gal as lactase and vice versa since lactase is an iso-enzyme of β-Gal.

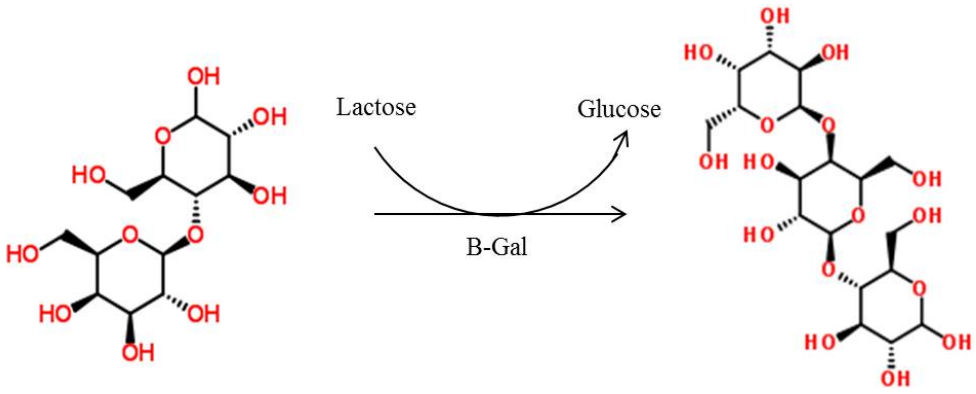


Figure 2.3: Transglycosylation of lactose to produce GOS, as shown for the example galactosyllactose.

2.3.1 Sources

Although β-Gal can be obtained from a variety of sources, their kinetic, chemical and reaction properties differ noticeably [21]. Microbial sources have some definite advantages over enzymes of plant and animal origin, since microorganisms are easier to handle, have a higher multiplication rate and produces high yields [9]. Currently there is a considerable amount of known β-Gal sources including species from 23 bacterial genera, 11 fungi genera and 4 yeast genera. Table 2.2 and Table 2.3, as summarized by Panesar *et al.* in 2010, includes all the known microbial sources of β-Gal. For the purpose of this study, only β-Gal from *E. coli* will be discussed in greater detail.

2.3.2 Structure of β-Gal from *Escherichia coli*

β-Gal (Figure 2.4) from *E. coli* is a large homotetramer with a total molecular weight of 464 kDa. Each subunit consists of 1023 amino acids and is 116 kDa in molecular weight. It is composed of 5 domains and the active site is a deep pocket with identified mono- and divalent cation binding sites [22]. This deep pocket of the active site is built around the central $(\alpha/\beta)_8$ barrel where other domains are also recruited to this area to bestow specificity for the disaccharide substrate [23]. Some details regarding the enzyme-substrate interactions of β-Gal and lactose have been investigated previously. There are three main amino acid residues that are said to be located to the active site i.e. Glu461, Met502 and Glu537 (Shown in Figure 2.5) [19]. These, and other active site residues, have been studied regarding their specific roles and interactions with substrate hydroxyls. Following this, it was suggested that Glu461 may act as a proton donor while Glu537 is the nucleophile [24–26]. Glu537 is then also the amino acid residue that was targeted during site directed mutagenesis by Dodd [4] to yield E537D β-Gal. It is, however, unfortunate that the large size of β-Gal has limited detailed investigations into structural information regarding β-Gal ligand binding [19].

Table 2.2: Bacterial sources of β-Gal reproduced as was summarized by Panesar *et al.* in 2010.

Sources	Microorganism (s)
Bacteria	Alicyclobacillis acidocaldarius subsp. Rittmannii Arthrobacter sp. *Bacillus acidocaldarius, B. circulans, B. coagulans, B.subtilis, B. megaterum, B. Stearothermophilus* *Bacteriodes polypragmatus* *Bifidobacterium bifidum, B. infantis* *Clostridium acetobutylicum, C. Thermosulfurogens* *Corynebacterium murisepticum* *Enterobacter agglomerans, E. cloaceae* *Escherichia coli* *Klebsiella pneumoniae* *Lactobacillus acidophilus, L. bulgaricus, L. helviticus, L.kefiranofaciens, L. lactis, L. sporogenes, L. themophilus, L. delbrueckii* *Leuconostoc citrovorum* *Pedicoccus acidilacticti, P. pento* *Propioionibacterium shermanii* *Pseudomonas fluorescens* *Pseudoalteromonas haloplanktis* *Streptococcus cremoris, S.lactis, S. thermophius* *Sulfolobus solfatarius* Thermoanaerobacter sp. *Thermus rubus, T. aquaticus* *Trichoderma reesei* *Vibrio cholera* *Xanthomonas campestris*

Table 2.3: Fungal and Yeast sources of β-Gal reproduced as was summarized by Panesar *et al.* in 2010.

Sources	Microorganism (s)
Fungi	*Alternaria alternate, A. palmi*
	Aspergillus foelidis, A. fonsecaeus, A. fonsecaeus, A. carbonarius, A. oryzae
	Auerobasidium pullulans
	Curvularia inaequalis
	Fusarium monilliforme, F. oxysporum
	Mucor meilhei, M. pusillus
	Neurospora crassa
	Penicillum canescens, P. chrysogenum, P. expansum
	Saccharopolyspora rectivergula
	Scopulariapsis sp
	Streptomyces violaceus
Yeast	*Bullera singularis*
	Candida pseudotropicalis
	Saccharomyces anamensis, S. lactis, S. fragilis
	Kluyveromyces bulgaricus, K. fragilis, K. lactis, K. marxianus

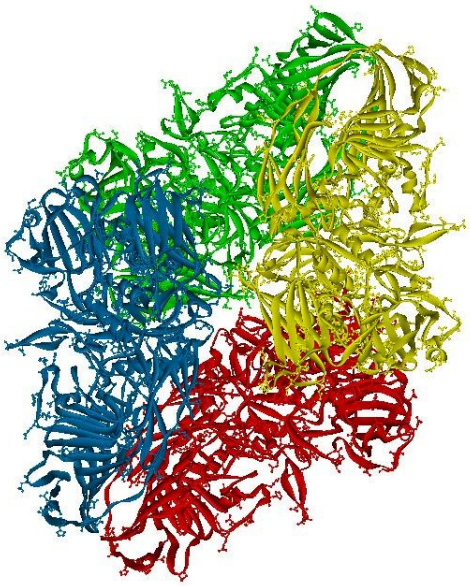


Figure 2.4: Three dimensional structure of the β-Gal enzyme. Secondary structure representation of the homotetramer in which each subunit is shown in a different colour. The figure was created using DS ViewerPro^{TM} Version 5.0.

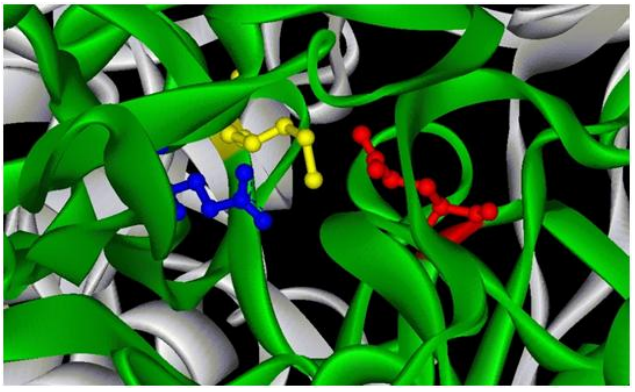


Figure 2.5: Three dimensional secondary structure representation of the active site of one of the subunits of β-Gal. Orientation of the amino acid residues located to a single active site are indicated in different colours i.e. Glu461 (Red), Met502 (Yellow) and Glu537 (Blue). The figure was created using DS ViewerPro^{TM} Version 5.0.

2.3.3 Applications of β-Gal

The enzymatic hydrolysis of lactose to the product monosaccharide sugars offers several benefits in three main areas i.e. health, environment and food technology. The hydrolysis of lactose can be carried out either by enzymatic catalysis with β-Gal that allows for milder pH and temperature operating

conditions or by acid treatment at more extreme conditions (150 °C) [27].

2.3.3.1 Health

As discussed previously, almost 70 % of the world's population has an inability to digest significant amounts of dietary lactose, also known as lactose intolerance. This well known lactase enzyme deficiency limits individuals with regards to the intake of dairy products, since consuming such dairy products will lead to mild discomfort. Sieber *et al.* [28] stated that by hydrolyzing the lactose with β-Gal to the more readily utilizable monosaccharide sugars, glucose and galactose, this problem of lactase deficiency could be circumvented [28]. This is then also currently the main application for β-Gal in the health industry.

During the enzymatic hydrolysis of lactose, GOS are formed at the same time. GOS are used as ingredients in prebiotic food and these compounds are indigestible, acting as dietary fibre [7]. GOS promote intestinal bifidobacteria growth, ensuring a healthy environment in the intestine and the liver [7]. By developing an inexpensive and effective GOS manufacturing process through the implementation of β-Gal, this enzyme can satisfy the growing demand for GOS production.

2.3.3.2 Environment

The dairy industry produces abundant amounts of a byproduct called whey containing large amounts of lactose and protein [8]. This waste can cause several environmental and economical issues if it is not disposed of properly. Problems arise especially due to the high chemical and biochemical oxygen demand, which lactose is associated with [8]. Guimaraes *et al.* [8] is of opinion that by implementing β-Gal in the hydrolysis of the lactose present in whey, the whey can be converted into a very useful product, sweet syrup, that can

be used in the confectionary, soft drink, dairy and baking industries thereby limiting its negative impact on the environment. Whey lactose can also be degraded by β-Gal for GOS manufacturing purposes.

2.3.3.3 Food Technology

Excessive lactose crystallization is a common occurrence in milk products that have high lactose content, such as frozen milks, condensed milk, whey spreads and ice-cream [7]. This usually results in a unpleasant gritty, sandy or mealy texture. According to Grosova *et al.*, β-Gal can be used to prevent this side-effect by processing such products to reduce the lactose concentrations to more acceptable values and by doing so, improve the sensorial and technological quality of these dairy foods.

Many of the above mentioned applications for β-Gal prevents the use of the free, unimmobilised form, due to the inability to effectively remove this still active enzyme from the product without turning to time-consuming methods. On the other hand, immobilised β-Gal, whether it is to glassplates [29], cellulose beads [30] or fibers [31], are readily manipulated and as a result, relatively simple to remove from crude mixtures. Strategies for the immobilisation of proteins are endless and since the covalent immobilisation of β-Gal forms part of this project, a detailed investigation into these strategies is necessary.

2.4 Protein Immobilisation

According to Rao *et al.* [32], the immobilisation of proteins in general has been performed successfully on packed beads, hollow fiber modules, magnetic nanoparticles, in hydrogels and on suspended particles. These immobilisation procedures can include physical or chemical adsorption of the protein to the solid support surface as well as random or site-specific orientation of the

immobilised protein.

Protein immobilisation is very attractive, especially for enzymatic studies, due to the proposed enhanced stability of the protein of interest as well as the extended range of applications the protein can now be used for. According to Panesar *et al.* [9], β-Gal may have numerous applications in the food and dairy industries, but currently the marginal stability of the enzyme hinders the general industrial implementation of this biocatalyst on a larger scale. β-Gal enzyme stabilization via immobilisation to solid supports and a variety of cross-linking strategies to ensure optimum subunit-subunit interactions as well as a stable multimeric form of the enzyme have been investigated by several research groups [33; 34]. The idea behind this strategy is to determine the proper experimental conditions to yield an active and stable immobilised enzyme for potential implementation as a biocatalyst in the food technology industry [33; 34]. Table 2.4 [29–31; 35–56] is a summary of all the different methods that have been investigated for the immobilisation of β-Gal compiled by Grosov'a *et al.* [7]. The percentage activity recovered for the specific method is also annotated.

The highest percentage of activity recovered (90 %) were achieved via covalent binding to porous silanised glass via glutaraldehyde cross-linking [29], while the carbodiimide coupling of β-Gal to alginate beads also resulted in a relatively high percentage activity recovered (76 %) [46]. Interestingly, the physical adsorption methods, thought to be less harsh than covalent binding methods, did not necessarily result in a higher percentage recovery of activity (Table 2.4).

2.4.1 Physical adsorption

This very simple enzyme immobilisation method is established through physical forces (Van der Waals forces) that occur between the biocatalyst and the surface of the water-soluble carrier. Other forces can be involved in the interac-

Table 2.4: Current literature available on the immobilisation of β-Gal from a variety of sources via different methods as compiled by Grosov'a *et al.* in 2008 [7].

Source of enzyme	Immobilisation method	Recovery of activity (%)
K. fragilis	covalent binding on corn grits	8
	covalent binding on cellulose beads	82
	covalent binding on porous silanised glass modified by glutaraldehyde	90
	entrapment in alginate-carrageenan gels	
	adsorption on phenol-formaldehyde resin	23
	adsorption onto bone powder	83
K. lactis	covalent binding onto glutaraldehyde-agarose	36 - 40
	covalent binding onto thiosulfinate-agarose	60
	covalent binding on graphite surface	0.01
K. marxianus	covalent binding on oxides supports: alumina, silica, silicated alumina	< 5
E. coli	covalent binding onto glutaraldehyde-agarose	39
	covalent binding onto thiosulfinate-agarose	75 - 85
	entrapment in liposomes	28
	covalent binding onto gelatin cross-linking with chromium (III) acetate	25
	covalent binding onto gelatin cross-linking with glutaraldehyde	22
	adsorption on chromosorb-W	
B. circulans	adsorption onto a ribbed membrane made from polyvinylchloride and silica	
A. oryzae	fibers composed of alginate and gelatine cross-linking with glutaraldehyde	56
	carbodiimide coupling to alginate beads	76
	entrapment in a spongy polyvinyl alcohol cryogel	
	entrapment in cobalt alginate beads cross-linked with glutaraldehyde	83
	microencapsulation in alginate beads	64
	encapsulation into gelatin and cross-linking with transglutaminase	8 - 46
	adsorption on phenol-formaldehyde resin	54
	adsorption on polyvinylchloride (PVC)	
	adsorption on silica gel membrane	
	adsorption on celite	2
	covalent binding to chitosan	18.4
	cross-linked aggregation by glutaraldehyde	13.5
	covalent binding in polyurethane foams	
	covalent binding to the tysolated cotton cloth	55
A. niger	adsorption on a porous ceramic monolith	80
Chicken bean	immobilised on cross-linked polyacrylamide gel	72

tion between the biocatalyst and the carrier i.e. hydrogen bridges, heteropolar (ionic) bonds and hydrophobic interactions [57].

The simplicity of this method for biocatalyst immobilisation as well as the small influence it has on the biocatalyst conformation contributes to the main advantages of physical adsorption, especially for enzyme immobilisation [9]. It has, however, been shown that the simple process of physical adsorption immobilisation has some major drawbacks i.e. random attachment, relatively weak adsorptive binding forces between the carrier and the biocatalyst, decrease of immunological capture efficiency, protein denaturation, molecule accessibility problems as well as the occurrence of overlapping [58].

As summarized by Panesar *et al.* [9], several organic (starch, cellulose, ion-exchange resins and activated carbon) and inorganic (silica, alumina, ceramics, porous glass, clay, diatomaceous earth, bentonite, etc.) support materials can be used for the physical adsorption of enzymes. Refer to Table 2.4 for previous solid supports used in β-Gal physical adsorption strategies accompanied by the percentage activity retained, which provides a measure of the success of the method.

2.4.1.1 Immobilised metal-chelate affinity chromatography (IMAC) enzyme immobilisation

IMAC is a commonly known technique for the purification of proteins fuzed to poly-histidine (His) tags. In some cases the adsorption of this poly His-tag to the chelate support is quite strong and may further suffice as a medium for enzyme immobilisation. IMAC can therefore be seen as a physical adsorption technique for the immobilisation of poly-His tagged proteins.

As previously mentioned, physical adsorption has some drawbacks. The main drawbacks of IMAC include the reversibility of the binding process as well as the possible undesired release of metals to the reaction media [59]. These may become a problem when the immobilised protein complexes are

used for industrial purposes.

This is the method of immobilisation that Dodd [4] used. Due to the possible industrial implementation of this novel technique, the IMAC physical adsorption of β-Gal to the MNP surface is not preferred due to possible enzyme and metal leakages. To prevent these leakages form occurring, this study focusses on the covalent immobilisation of β-Gal to MNPs, which would ensure a strong, permanent bond between the protein of interest and the MNP surface.

2.4.2 Entrapment method

Entrapment can be easily explained as the enclosure of enzymes or molecules in a small space. The major methods of entrapment are membrane (including microcapsulation) and matrix entrapment [9].

According to Panesar *et al.* [9], the simplicity, especially when referring to the method to obtain spherical particles, is possibly the major advantage of this technique. Furthermore, the transparency and general mechanical stability of such beads formed from alginate may be advantageous in enzyme immobilisation studies [9]. The major drawback of this enzyme immobilisation technique is the potential slow leakage of enzyme during continuous use [9]. Certain improvements can, however, be made by means of appropriate linking procedures.

Some of the membranes that are commonly used for enzyme immobilisation through entrapment are cellulose, nylon, polyacrylamide and polysulfone as mentioned by Panesar *et al.*. Matrices on the other hand can consist of more polymeric materials such as agar, Ca-alginate, k-carragenin, collagen and polyacrylamide or even some solid matrices such as porous ceramic, activated carbon and diatomaceous earth [9].

2.4.3 Cross-linking

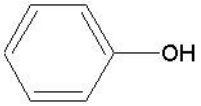
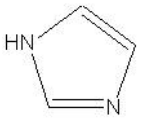
Cross-linking, used on its own or in combination with other immobilisation techniques, such as entrapment and adsorption, is a well-known method for biocatalyst, protein and peptide immobilisation to either other protein molecules or to functional groups on insoluble carrier/support materials [60]. Cross-linking can be via ionic or covalent bonds resulting in physical or oxidative cross-links. Cross-linking reagents have reactive ends to specific functional groups i.e. sulfhydryls, primary amines, etc [60]. The reactions can include cross-linking compounds that are bi-functional (homo- or hetero-bi-functional) or even multi-functional. Some examples of reactive cross-linker groups with their functional group targets are: hydrazide (carbohydrate), imidoester (amine), carbodiimide (amine/carboxyl), isocyanate (hydroxyl), carbonyl (hydrazine), maleimide (sulfhydryl), NHS-ester (amine), vinyl sulfone (sulfhydryl, amine, hydroxyl), PFP-ester (amine) and hydroxymethyl phosphine (amine) [60].

2.4.4 Covalent enzyme immobilisation

A covalent bond is defined as a chemical link between two atoms in which electrons are shared between them. Some amino acid groups can take part in covalent immobilisation i.e. ε-amino of lysine and N-terminal amino group, sulfhydryl of cysteine, carboxyl group of aspartate and glutamate and C-terminus carboxyl group, phenolic of tyrosine and imidazole of histidine [32]. A wide range of attachment methods exist. These, with their accompanying reactive amino acid groups, are summarized in Table 2.5.

According to Teste *et al.*, covalent anchoring of biomolecules enable stronger and more reproducible attachment to solid supports compared to randomized or physical adsorption [58]. Additionally, covalent immobilisation can produce molecules arranged in a defined, orderly fashion and also allows the possible use of linkers and spacers to help minimize steric hindrances that can occur

Table 2.5: Amino acids involved in covalent immobilisation, and method of attachment as summarized by Rao *et al.* in 1998 [32].

Amino acid	Reactive residue	Method of attachment
ϵ-amino group of lysine and N-terminal amino group	-NH2	Diazotization Peptide bond formation Arylation Alkylation Schiff-base formation Amidination
Sulfhydryl of cysteine	-SH	Alkylation Thio-disulfide interchange Mercury enzyme interaction
Carboxyl group of aspartate and glutamate and C-terminus carboxyl group	-COOH	Peptide bond formation
Phenolic of tyrosine		Diazotization
Imidazole of histidine		Diazotization

between the protein and immobilisation surface [61]. This is advantageous, since biomolecule-solid support complexes and conjugates are used in analytical, clinical, environmental, biomedical and industrial chemistry where the procedures require the permanent or semi-permanent immobilisation of functional biomolecules.

Apart from forming strong linkages between the protein and the solid support, covalent immobilisation can potentially result in changes in the structural configuration of the immobilised protein. This is, according to Camarero [61], "mainly due to the heterogeneous chemical nature of proteins as well as the marginal stability of the native, active tertiary structure over the denatured, and inactive random coil structure". In the case of enzymes, such a struc-

tural change can lead to inaccessibility of the active site, reduced activity or even altered reaction pathways [32]. It is noteworthy that all of these can be avoided through an in-depth study of the primary and secondary structure of the protein of interest.

Gauthier and Klok [62] is of opinion that, for an enzyme to be functionally immobilised covalently to a solid support, the amino acid residues targeted have to be reasonably exposed on the surface of the protein of interest and accessible. This requires an investigation into the tertiary structure of the protein as well as information regarding the average surface accessibility of these targeted amino acid residues [62]. Camarero [61] feels that it is also important to consider that the immobilisation reaction should be able to occur efficiently under physiological conditions to avoid protein denaturation during the coupling step.

Covalent protein immobilisation is usually achieved through two steps i.e. surface activation and biomolecule immobilisation (adsorption through an incubation step) [58]. Furthermore, to achieve site-specific protein immobilisation, two unique and mutually reactive groups are required on both the solid support and protein surface [61]. To conclude, covalent immobilisation can produce a permanent and reproducible active immobilised enzyme with the active site still accessible to the substrate and where steric hindrance is limited to a minimum through the use of a linker.

There exists a wide range of solid supports for the immobilisation of proteins. The selection of a specific solid support relies on the functionality and separation properties of the support as well as the final application of the immobilised protein. As part of this project, the immobilisation of a protein to magnetic nanoparticles, it would be beneficial to elaborate on this aforementioned solid support.

2.5 Magnetic nanoparticles (MNPs)

Nanoparticles, and other nanomaterials, have recently turned into a popular topic with regards to biotechnological and other scientific applications where the immobilisation of different biomolecules to these materials has been demonstrated [63]. Proteins that are conjugated to these nanoparticles can be used for the studying of protein-protein interactions, screening of combinatorial libraries, drug delivery, and enzyme immobilisation for polysaccharide hydrolysis [63]. According to Tartaj *et al.* [64], these nanoparticles with dimensions between molecules and micro-sized structures are small enough that they have unique properties but are sufficiently large that they provide access to realms of quantum behaviour.

According to Tartaj *et al.* [64], there are four advantages in using particles with a size smaller than 100 nm:

- higher effective surface areas
- improved tissular diffusion
- lower sedimentation rates
- reduced magnetic dipole-dipole interactions.

MNPs, as compared to just nanoparticles, are particles with strong magnetic properties. These have become particularly useful, firstly because of the capability of magnetic targeting (positioning to a specific area) and secondly because they are readily manipulated, controlled and recovered for reuse by an external magnetic field. Some of the unique characteristics of MNPs are high field irreversibility and superparamagnetism [64]. Potential applications of MNPs include biomedicine [65] where superparamagnetic behaviour is necessary at room temperature, data storage [66] where the particles need to have a switchable, stable magnetic state, catalysis [67], magnetic particle imaging [68], magnetic resonance imaging [69] and environmental remediation [70].

2.5.1 Magnetic core material

Depending on the *in vivo* or *in vitro* application of the MNPs, several factors need to be taken into consideration when choosing the magnetically responsive component. These factors include the toxicity, magnetic properties, final particle size as well as the biocompatibility of the material considered for the particle core [64]. For the *in vivo* application of these particles, a biocompatible polymer coating is also necessary to prevent changes from the original structure, formation of aggregates and biodegradation [64].

According to Tartaj *et al.* [64], some common materials used for the magnetic component are iron, magnetite, cobalt, nickel, samarium-cobalt and neodimium-iron-boron. The iron oxides, magnetite (Fe_3O_4) or its oxidized form maghemite (Fe_2O_3), are the most commonly employed material to produce MNPs, especially for biological and biomedical applications. These magnetite MNPs are easy to synthesize, have strong magnetic properties [71] and exhibit low toxicity [72], which will facilitate future application in the food process industry. In contrast to this, cobalt and nickel, that also has high magnetic properties, are toxic and susceptible to oxidation and not often used for MNP synthesis [64].

2.5.2 Synthesis of MNPs

The ultimate synthesis/preparation method for these particles are those that allow the synthesis of particles that are nearly uniform in size and shape. Figure 2.6 is a flow diagram that summarises all the synthesis methods for MNPs [64]. The simplest of these is the coprecipitation method to yield magnetite particles and this is the only method that will be discussed here in detail.

Magnetite nanoparticle synthesis can occur through a coprecipitation reaction where magnetite (Fe_3O_4) particles form spontaneously when an aqueous solution of ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions is mixed with a strong base like NH_4OH/NaOH at room temperature. It has, however, been shown that to

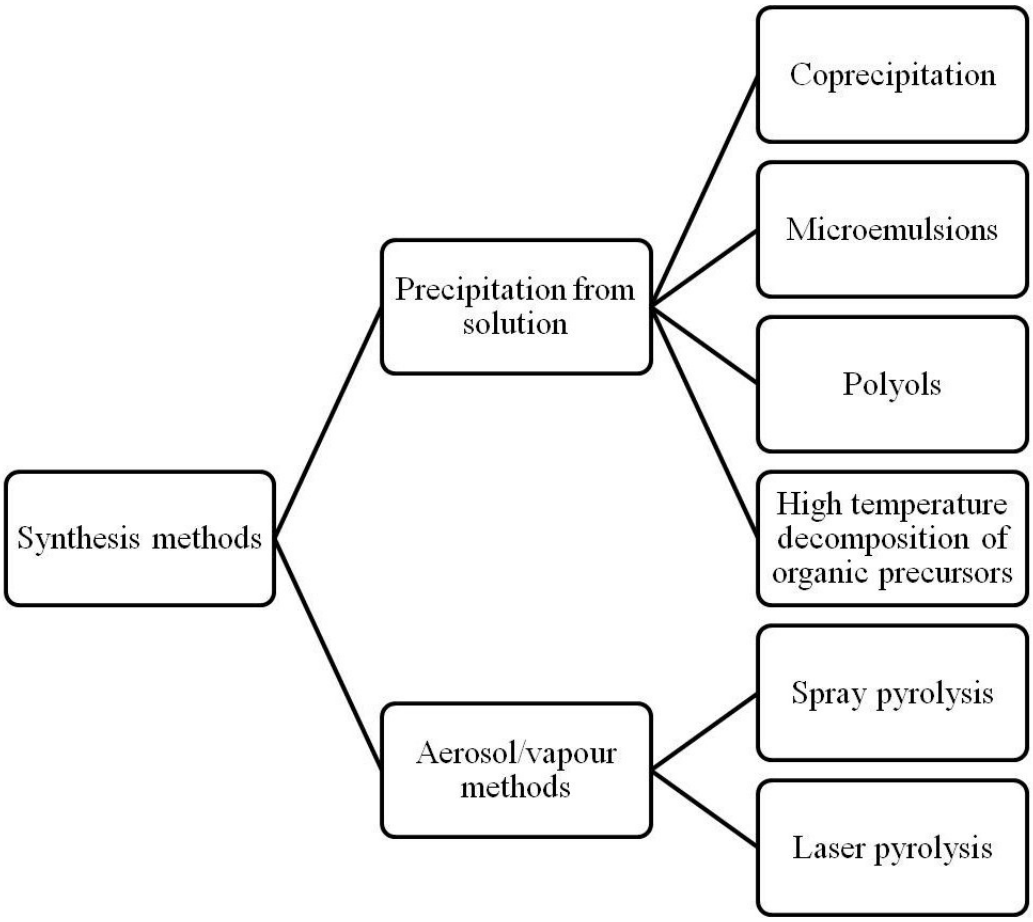


Figure 2.6: Flow diagram explaining the different precipitation and aerosol methods for the synthesis of MNPs [64].

narrow the size distribution to yield uniform physical and chemical properties is not an easy task [73]. Different techniques to optimize different properties of magnetite nanoparticles exist but most of these are rather conflicting.

Zhu and Wu [74] proposed that they could produce nanoparticle synthesis results with an average particle size less than 6.0 nm by applying forced mixing without any surfactants. Sun and Zeng [73] on the other hand stated that they can control the size of magnetite nanoparticles (3 to 20 nm) produced through simple organic phase synthesis. Both of these research groups completed the synthesis under nitrogen and at elevated temperatures. Yazdani and Edrissi [75] studied the effect of pressure on the size of produced nanoparticles and found that it had a significant effect. They found that when increasing the pressure of the nanoparticle synthesis reaction from 300 to 6000 mbar,

the obtained particle size increases from 8.3 to 16.8 nm at room temperature (25^{o}C) [75]. They attributed this phenomenon to physical property changes regarding the nanoparticles' large surface-to-volume ratio [75]. Liu *et al.* [76] employed high temperatures, stirring of the reaction mixture and a nitrogen atmosphere for nanoparticle synthesis. However, they used a surfactant (oleic acid) to form a waterproof shell around the magnetite nanoparticles. They report a simple yet efficient method to prepare Fe_3O_4 nanoparticles as a magnetic gel that has super paramagnetic properties at room temperature. The same authors also claim that these hydrophobic gels could facilitate the incorporation of magnetite nanoparticles in polymer matrixes through hydrophobic interactions between the compounds [76].

To conclude, it seems as though thermal treatment, synthesis under nitrogen as well as surfactant coating of the magnetite nanoparticles yields a superparamagnetic, concentrated hydrophobic gel that may perhaps be extremely versatile regarding surface functionalizations; especially with regards to polymer matrix coatings [76].

2.5.2.1 Properties of magnetite MNPs [77]

Magnetite is a black, ferromagnetic mineral at room temperature and has a Curie temperature of 850 K containing both divalent (Fe^{2+}) and trivalent (Fe^{3+}) iron. Magnetite has an inverse spinel structure and a cubic crystal system.

The surface area of magnetite ranges from 10 m^2g^{-1} for Fe_3O_4 formed by reduction of haematite to close to 100 m^2g^{-1} for particles ca. 50 nm across produced by precipitation. In 1966 Mannweiler [78] used three different methods to determine the surface area of a sample of cubic magnetite crystals (194 nm across) that was obtained by the reduction of haematite. These were X-Ray diffraction line broadening, Transmission Electron Microscopy (TEM) and BET (N2) measurement. The resulting areas obtained by all three methods

were in very good agreement - 5.6–6.6 m^2g^{-1}.

In general magnetite has a very high surface to volume ratio, which makes it ideal for surface chemistry modifications.

For the final implementation of our novel technique in the food processing industry, we need to consider different bioreactors that might facilitate the application of our MNP immobilised β-Gal enzyme.

2.6 Bioreactors

There are three general bioreactor systems, the difference depending on the method of biocatalyst immobilisation and the type of process required. These are Fluidised bed reactor (FBR), Packed bed reactor (PBR) and Membrane reactor (MR).

2.6.1 Fluidised bed reactor (FBR)

In this type of bed reactor the immobilised enzyme particles are fluidised, which means that the flow of the substrate stream causes the particles to become suspended in the substrate stream. If their density is sufficiently high the immobilised enzyme particles are quite small, ranging from 20 to 40 μm in diameter [79].

The main advantage of FBR over PBR is that pretreatment of the feed is not necessary before the use. However, FBR is usually quite difficult to scale up and its use is commonly restricted to smaller scale but high priced products [79; 80].

2.6.2 Packed bed reactor (PBR)

This bioreactor system is the most popular for the enzymatic hydrolysis of lactose [81]. In this type of system a column is packed with the immobil-

ised enzyme particles and the accompanying substrate is pumped through the column in plug flow direction [7]. This plug flow is defined as the constant velocity of flow in every part of the system and this type of reactor improves contact between the two phases, liquid and stationary. The matrix used is normally fairly rigid with the diameter of the pellets about 1–3 mm.

An advantage of the PBR is that it permits the use of high density biocatalysts and therefore results in high volumetric efficiencies. These type of reactors are also preferred in enzymatic reactions involving product inhibition, e.g. the enzymatic hydrolysis of lactose.

The limitation of the PBR is that it does not allow for the monitoring and regulation of pH and temperature in reactors with a >15 cm diameter. According to Lilly and Dunnill [82], it is more advantageous to have a uniform pellet size and an upward substrate stream flow.

2.6.3 Membrane reactor (MR)

A MR system consists of a membrane (e.g. dialysis membrane) that is immersed into a stirred tank. The membrane contains the enzyme of interest, generally in a free form, within an inner chamber where the substrate is allowed to freely move in and the product of the enzymatic reaction, allowed to move out [7].

This reactor allows for continues operation with high enzyme concentration and low pressure [7]. It has, however, been shown that the enzyme in a MR has less stability than the PBR enzyme due to wash out effects. There is also a continuous need for membrane replacement and a limitation to the diffusion of substrate and products through the membrane [83].

Of these three general bioreactor systems, PBR are the most often used for lactose hydrolysis. The use of MNPs in this study may require the development of a bioreactor where several incubation periods of the same batch of milk with the protein coated particles could be used to remove the desired amount of

lactose under controlled conditions.

2.7 Conclusion

β-Gal from *E. coli* was used in this study since *E. coli* is a well characterised organism and easier to work with as compared to human lactase. Due to the biotechnological advances and applications in industrial processes and biomedicine, MNPs was chosen as the desired solid support simply because of the capability of magnetic targeting, surface modifications and its simplicity. The covalent strategy for the coupling of β-Gal to MNPs is mainly to prevent enzyme leakage, which can readily occur with physical adsorption, non-covalent, enzyme coupling.

For the ultimate bioreactor application of the immobilised enzyme-particle complex, a functional and covalently linked β-Gal MNP compound needs to be assembled. To achieve this, a pure form of the enzyme as well as functionalized MNPs are necessary.

During the subsequent chapters, the individual aims of this project are investigated and discussed to ultimately explain the properties and possible industrial implementation of this novel technique to enhance current practice in producing lactose free dairy products.

Chapter 3

PROJECT CONTROLS: β-GALACTOSIDASE CHARACTERIZATION

3.1 Introduction

In order to achieve the main objective of this study, the covalent immobilisation of β-Gal to commercially available MNPs for the possible application of binding lactose and physically removing this from milk, functional β-Gal had to be obtained. Functional β-Gal from *E. coli* was previously cloned and successfully purified by Dodd [4]. It was, however, necessary to repeat and verify some of this previous work regarding the β-Gal mutagenesis, expression, purification and activity to confirm the reproducibility of the results as well as to compare different strategies for β-Gal enzymatic activity determination. Here, results of a microtitre plate assay was compared to the results previously obtained by Dodd with a test tube assay. This was necessary since the new microtitre plate assay [5] limits the influence of human error and therefore provides better and faster control over the assay conditions.

By going through these steps to obtain the pure and active wildtype and

E537D His_6-β-Gal, the significantly lower enzymatic activity of the pure mutant β-Gal would be verified that would theoretically allow for the removal of lactose from milk when immobilised to MNPs with minimal hydrolysis to glucose and galactose (refer to section 1.3). In addition to this, the pure mutant β-Gal obtained would be utilized in the succeeding immobilisation approaches.

The cloning and mutagenesis of the *LacZ* gene was previously performed by Dodd [4]. The Gram negative bacterium, *Escherichia coli*, was used in this study as the model organism for the heterologous production of β-Gal. According to Baneyx [84], *E. coli* is one of the most attractive systems for the production of heterologous proteins due to the well characterized nature of its genetics, availability of a whole range of cloning vectors and its ability to grow rapidly and at high density on inexpensive substrates. Furthermore, growing of cultures and inducing heterologous protein expression with *E. coli* as the host is relatively straight forward, resulting in sufficient protein yields for protein purification and subsequent analyses. Although *E. coli* is not generally regarded as safe, especially not in the food process industry, it is an ideal model organism to form part of this proof of concept study.

In order to characterize any protein, a pure fraction is needed. Protein purification is generally directed at exploiting protein characteristics like size, isoelectric point, structure or biological activity to isolate a specific single type protein from a mixture or crude extract of proteins for subsequent use. Purification methods include a range of chromatographic methods like ion-exchange, size exclusion, affinity and high performance chromatography. Heterologous expression of recombinant proteins, however, allows for the inclusion of a histidine tag to the N- or C-terminal of the required protein. This modification of the primary structure of the protein yields an active protein that can be readily isolated by IMAC.

IMAC was first introduced in 1975 and is directed at exploiting the chemical affinity that exists between solute molecules and immobilized metal ions [85].

The concept of IMAC can be explained as metal-ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} and Fe^{2+}) localized in an exposed position in an insoluble polymer matrix (eg. carboxylic-sulfonic, or sulfate ester-type cation exchangers) that has affinity for certain analytes or ligated tags on the N- or C-terminal of the protein of interest in the sample to be fractionated [85]. IMAC is widely used for the purification of poly-histidine tagged proteins since these poly-histidine tags can readily interact with immobilised transition metals and separate these tagged proteins from other proteins present in the crude extract by means of gradient elution. According to Porath [85], some of the main advantages of IMAC are the mild and non-denaturing conditions it allows for the isolation of a specific protein of interest on a milligram or gram scale. IMAC consists primarily of five consecutive operations i.e. column equilibration, sample injection, removal of non-specific bound fraction, elution of protein of interest and regeneration [85]. Due to the His_6-tag cloned on the N-terminal of the wildtype and mutated versions of β-Gal [4], an IMAC purification strategy was used to obtain pure protein for experiments carried out in this study.

After the isolation of the protein of interest, protein characterization is necessary. The kinetic parameters of β-Gal from *E. coli* have been investigated by several research groups using various substrates, such as: o-nitrophenyl-β-D-galactopyranoside (ONPG), 4-nitrophenyl β-D-galactopyranoside (PNPG), allolactose and alpha-lactose, reaction temperatures, incubation volumes, buffer compositions and the wavelengths at which the absorbencies of the reaction products were measured. ONPG was most commonly used as substrate with the reaction temperature varying between 25 ^{o}C [5], 30 ^{o}C [BRENDA database (BRaunschweig ENzyme DAtabase)(EC 3.2.1.23)] and 37 ^{o}C [86], while the reaction was performed in test tubes (3 ml; [86]) or microtest plates (200 μl; [5]) and monitored at 410 nm [86] or 420 nm [5].

Yuan *et al.* [87] investigated the effect on enzymatic activity of several mutations of the glutamic acid (E) in position 537, a residue that was also

targeted by Dodd [4]. When this glutamic acid was mutated to an aspartic acid (D), a 760 fold decrease in activity was found. Mutation to a glutamine, however, resulted in a 160 000 fold decrease in activity while a valine in position 537, instead of the glutamic acid, caused a 28 000 fold decrease in activity with ONPG as substrate at 25 ^{o}C [87]. The accompanying, calculated K_m values for the individual point mutations were found to be similar to that of the wildtype enzyme except for the E537D mutation, where a slightly higher substrate concentration was required to reach half maximal velocity [87]. For the purpose of this study, the ideal would be a mutated enzyme with significantly less activity when compared to the wildtype enzyme, but one that still offers both the very slow hydrolysis of lactose, to compensate for the possible loss in sugar content, as well as sufficient substrate binding to enable the binding and removal of lactose from milk. The point mutation of E537D was therefore selected for the purpose of this study [4].

According to a consumer sensory test, conducted by Adhikari *et al.* [18], commercially available lactose-free milk is sweeter, contains more cooked and processed flavours and has a higher viscosity and a chalkiness when compared to normal low fat milk. The sweeter taste of lactose-free milk results from the relative sweetness of glucose and galactose (hydrolyzed lactose) compared to lactose itself. The use of the E537D mutant β-Gal would, therefore, theoretically allow the removal of a percentage of the lactose present in milk, whereafter only the residual lactose would be hydrolyzed to glucose and galactose, by using the immobilised wildtype β-Gal, instead of all the lactose, as is current practice [18]. This will limit the ultimate sweetness of the final lactose free milk and possibly improve the sensory quality.

3.2 Materials and Methods

3.2.1 Reagents and chemicals

The plasmid vector pTrcHis-*LacZ* and mutagenesis product, pTrcHis-*LacZ_E537D*, were obtained from previous studies in this laboratory. A bicinchoninic acid (BCA^{TM}) protein assay kit was purchased from *Thermo Scientific* (Rockford, USA) and microplates for protein determination assays from *Heinz Herenz Hamburg* (Germany). Precision Plus Protein Kaleidoscope^{TM} markers for SDS PAGE and immunoblotting were purchased from *Bio-Rad Laboratories, Inc.* (Hercules, USA). Anti-β-galactosidase primary antibody, as a rabbit IgG fraction, was purchased from *Invitrogen* (USA), while Alkaline Phosphatase conjugated secondary anti-rabbit IgG, produced in goat, and sample buffer according to Lämmli (2X) were purchased from *Sigma-Aldrich Chemical Co.* (Germany). HisProbe^{TM}-HRP as well as Pierce ECL Western Blotting substrate were purchased from *Thermo scientific*. HiTrap^{TM} (1 ml and 5 ml) chelating columns (*Amersham Biosciences*) and Spectra/Por Dialysis Membrane with M_w cut off: 6-8000 (*Spectrum Laboratories, Inc.*) were obtained from a local supply house. 2-nitrophenyl-β-D-galactopyranoside (ONPG) was obtained from *Sigma-Aldrich Chemical Co.* (Germany). All chemicals used for the preparation of buffers and solutions were of analytical grade or higher and purchased from *Merck (Pty) Ltd* (South Africa), *Sigma-Aldrich Chemical Co.* (Germany) or *Bio-Rad Laboratories (Pty) Ltd* (South Africa). An Ultra Pure Milli-Q water system (*Millipore* (South Africa)) was used to produce reagent grade water used in all buffers and experiments.

3.2.2 Wildtype and E537D β-Gal expression and verification

3.2.2.1 Protein expression

Starter cultures (SC) were prepared from pTrcHis-*LacZ* and pTrcHis-*LacZ_ E537D* *E. coli Top 10* freezer stocks by inoculating 5 ml Luria-Bertani (LB) media containing 50 ug/ml ampicillin overnight at 37 ^{o}C with shaking (@ 230 rpm). The SC was subsequently diluted 50X in fresh LB media containing antibiotic (100 ug/ml ampicillin) and grown at 37 ^{o}C to an OD_{600} = 0.6. β-Gal protein expression was subsequently induced by the addition of isopropyl-1-thio-B-d-galactopyranoside (IPTG) (0.3 mM). An uninduced control culture was included for every protein sample. The induced and uninduced cultures were subsequently incubated at 37 ^{o}C for 5 hours with shaking (@ 235 rpm) to allow expression of proteins. Cultures were then centrifuged (4000 x g, 4 °C for 20 minutes), the supernatant discarded and the wet cell weight determined. The pellets were resuspended in ice cold lysis buffer (50 mM phosphate, pH 8, 300 mM NaCl, 10 mM Imidazole, 5 mM sodium metabisulphite and 0.1 % NP40), 10 ml per 1 g cells. Phenylmethanesulfonylphosphonate (PMSF) was added to a final concentration of 10 mM as a protease inhibitor. The lysate was sonicated on ice (1 min bursts, pulse mode, 50 % duty cycle) before incubation at 4 ^{o}C for 30 minutes. Thereafter, the lysate was centrifuged (20 000 x g, 4 ^{o}C for 20 minutes), the supernatant collected, which contains the protein of interest, and the pellet resuspended in lysis buffer (20 ml) containing PMSF (10 mM). The presence of the wildtype and E537D His_6-β-Gal protein was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), HisProbe and immunoblot analyses.

3.2.2.2 Protein determination

Protein standards, ranging from 0 to 2.0 mg/ml with a working range between 20–2000 μg/ml, were prepared using the BCA method according to manufacturer's instructions. Bovine serum albumin (BSA) was used as a protein standard and a standard curve was constructed from which the unknowns were determined.

3.2.2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

Preparation and Casting of the resolving and stacking gel (12 % T, 2.6 % C resolving gel and 4 % T, 2.6 % C stacking gel)

SDS PAGE was performed as described by Laemmli [88]. Resolving gel buffer (0.375 M Tris, pH 8.8, 0.1 % (m/v) SDS), (7 ml) was mixed with Acrylamide stock solution (3 ml, 40 % (w/v)), 10 μl N,N,N'N'-tetramethilene diamine (TEMED) and 100 μl of freshly prepared 20 % (m/v) Ammonium persulfate solution and the gel was cast directly afterwards. A small volume of deionized water was pipetted on top of the gel to even the gel front and removed after the gel had polymerized (20 minutes). Stacking gel buffer (0.126 M Tris, pH 6.8, 0.1 % SDS, m/v) (4.5 ml) was mixed with Acrylamide stock solution (0.5 ml, 40 % (w/v)), 5 μl TEMED and 50 μl of freshly prepared 20 % (m/v) Ammonium persulfate solution and the gel poured. A 10 well comb was inserted and the gel allowed to polymerize for 40 minutes before sample application.

Sample and Kaleidoscope^{TM} marker preparation

Each protein sample as well as a Rainbow Kaleidoscope^{TM} marker was treated with an equal volume of sample/treatment buffer (0.004 % bromophenol blue in 0.125 M Tris, 4 % (m/v) SDS, 20 % glycerol, 10 % (v/v) 2-mercaptoethanol, pH 6.8) and boiled for 10 minutes at 90 ^{o}C. The samples were then kept on ice until loaded onto the gel.

Electrophoresis

The gel was assembled as per the manufacturer's instructions. The inner and outer chamber of the electrophoresis tank were filled with electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1 % (m/v) SDS, pH 8.3), and the protein samples were loaded. Electrophoresis was performed at 4 °C and a constant voltage of 200 V.

Staining and destaining procedures

The gel was stained for 1 hour in Coomassie blue staining solution (0.125 % (m/v) Coomassie Brilliant Blue R250, 50 % (v/v) methanol and 10 % (m/v) acetic acid). The staining solution was decanted and the gel destained in destain solution 1 (50 % (v/v) methanol and 10 % (v/v) acetic acid) overnight followed by destain solution 2 (5 % (v/v) methanol and 7 % (v/v) acetic acid).

3.2.2.4 Immunoblot and HisProbe blot analyses

Electro-transfer

Proteins were separated by SDS PAGE as described in section 3.2.2.3 and transferred to a nitrocellulose membrane as described by Strott [89] and per manufacturer's instructions for the transfer equipment. Electro-transfer of proteins to the nitrocellulose membrane was performed at ambient temperature with a constant voltage of 110 V for 90 minutes with the electro-transfer tank filled with transfer buffer (50 mM Tris, pH 8.3, 192 mM glycine and 20 % (v/v) methanol).

Immunoblot analysis

The membrane was blocked with casein buffer (15 ml, 10 mM Tris, pH 7.6, containing 0.15 M NaCl, 0.5 % casein and 0.02 % thiomersal) at 4 °C overnight while agitating. The blot was washed 3 times with Tris buffered saline tween (TBST) (15 ml, 25 mM Tris, pH 7.6, 0.15 M NaCl and 0.05 % Tween-20) for 5 minutes before adding the primary antibody. A rabbit anti-β-Gal serum was diluted, 1 in 100 000, in 2 % BSA/TBST (prepared from a 10 % BSA stock

solution) as primary antibody and incubated with the membrane for 1 hour at room temperature while agitating. Before adding the secondary antibody, the blot was washed again several times with TBST. As secondary antibody, an Anti-Rabbit IgG, Alkaline Phosphatase conjugated antibody was diluted, 1 in 30 000, in 2 % BSA/TBST and incubated with the blot for 1 hour at room temperature. The blot was subsequently washed 3 times with TBST before the substrate solution was added. As substrate solution a combination of 33 μl BCIP-T (50 mg/ml in dimethylformamide) and 44 μl NBT (75 mg/ml in 70 % dimethylformamide) in 10 ml Alkaline Phosphatase (AP) buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 10 mM $MgCl_2$) was prepared and incubated with the blot to yield a colour reaction on the blot.

HisProbe blot analysis

The membrane was blocked with 2 % BSA/TBST (15 ml) at 4 ^oC overnight while agitating. Afterwards, the blot was washed with TBST for 5 minutes before adding the HisProbe^{TM}-HRP. This is a high specificity probe consisting of horseradish peroxidise (HRP) coupled with nickel as probe specific for the His_6-tag cloned on the N-terminal of proteins (Figure 3.1) [90].

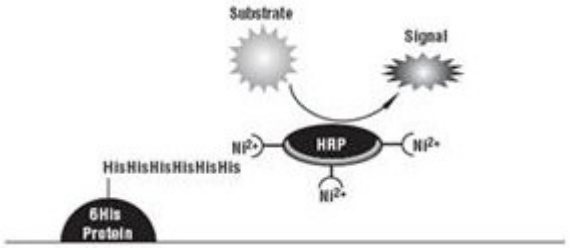


Figure 3.1: His-probe consisting of HRP coupled with nickel, used for the detection of His_6-tagged proteins, His_6-β-Gal in this study [90].

The HisProbe^{TM}-HRP was diluted, 1 in 6000, in 2 % BSA/TBST and incubated with the blot for 1 hour at room temperature. The blot was subsequently washed 4 times with TBST for 4 minutes before the substrate solution was added. As substrate, the Pierce ECL Western Blotting substrate kit was used as an enhanced chemiluminescent substrate for the detection of

HRP, prepared by the mixing of detection reagents 1 and 2 in a 1:1 ratio. Chemiluminescence was used to verify the presence of the protein.

3.2.3 Wildtype and E537D His_6-β-Gal IMAC purification, dialysis and verification

3.2.3.1 IMAC

The supernatant samples of the expressed wildtype and E537D His_6-β-Gal were purified by affinity chromatography using HiTrap^{TM} chelating columns and an AKTA Prime system [91]. The chelating sites of the columns were saturated with a 100 mM $NiSO_4$ solution and following this were equilibrated with 9 column volumes of wash buffer (50 mM phosphate, pH 8, 300 mM NaCl, 20 mM Imidazole, 25 mM β-mercaptoethanol, 5mM sodium metabisulphite, 10 % glycerol). The filtered protein sample (e.g. wildtype His_6-β-Gal) was loaded on the column followed by washing with 2 column volumes of the wash buffer to remove proteins that bound non-specifically. The protein was eluted using 8 column volumes of an imidazole gradient from 0 to 0.5 M. The same protocol was used for the E537D His_6-β-Gal. Tween-20 and sterile deionised water was added to the fraction collection tube just before the purified protein sample was collected, which is current practice in our laboratory [4] to prevent the aggregation of the hexahistidine tags as well as to ensure solubility of the purified His_6-β-Gal.

3.2.3.2 Dialysis

Dialysis membrane (32 mm width with 20.4 mm diameter) (Spectra/Por) was used together with a phosphate buffered saline (PBS) buffer, pH 7.4 containing 0.2 % Tween-20, to remove the imidazole and β-mercaptoethanol from the purified protein samples that will interfere with downstream analyses. Afterwards, the presence of purified His_6-β-Gal was confirmed by SDS PAGE,

HisProbe and immunoblot analyses (refer to sections 3.2.2.3 and 3.2.2.4).

3.2.4 Enzymatic activity assays of wildtype and E537D β-Gal with ONPG

β-Gal activity assays were performed according to Sanbrook *et al.* [92] as adapted by Held [5]. A 20 mM stock solution of the ONPG substrate in 2X reaction buffer (200 mM sodiumphosphate, pH 7.0; 2 mM $MgCl_2$; 100 mM β-mercaptoethanol and 1.33 mg/ml ONPG in deionised water) was prepared and a dilution series from 0 to 16 mM was made using 2X reaction buffer without ONPG as diluent with 8 different concentrations in total. Aliquots of 100 μl for each dilution of ONPG were pipetted into the wells of a 96-well microplate in replicates of 6. The reaction was initiated with the addition of either 100 μl of the purified wildtype fraction (500X diluted) in triplicate or pure E537D β-Gal fraction in triplicate, diluted in milliQ water if necessary. After the addition of the purified protein the kinetic readings were initiated immediately with absorbance determinations made every 5 seconds for a total of 5 minutes at 37 °C. All absorbance determinations were made at 420 nm using a PowerWave^{TM} HT Microplate Spectrophotometer (BioTek Instruments) with the reader controlled by Gen5 Data Analysis Software. Data was analyzed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3.3 Results and Discussion

3.3.1 SDS-PAGE and Western blotting verification after protein expression

Wildtype and E537D His_6-β-Gal proteins were expressed as previously described. Each sample yielded both a supernatant and pellet sample after the

final centrifugation step.

To verify the presence of β-Gal in the expressed protein samples as well as the His_6-tag cloned on the N-terminal of β-Gal, all samples were analyzed through SDS PAGE, immunoblot and HisProbe blot analyses (Figure 3.2). β-Gal from *Aspergillus oryzae* was included in the above analyses as a positive control for untagged β-Gal.

SDS PAGE and immunoblot analysis (Figure 3.2(a) and (b)) confirmed that β-Gal was expressed and present in both the supernatant and pellet samples due to the corresponding size of the expressed proteins as well as the specificity of the β-Gal primary antibody used. β-Gal occurs naturally as a 464 kDa homotetramer, but should reveal a band corresponding to 116 kDa (monomer) when analyzed through SDS-PAGE. The slight size difference visible between the control β-Gal (116 kDa) sample (Lane 2, Figure 3.2) and the expressed β-Gal (120 kDa) sample (Lanes 3–10, Figure 3.2) is due to the His_6-tag cloned on the N-terminal of the protein that adds approximately 5 kDa to the protein size. It is also clear from the data presented in Figure 3.2(a) that there is a significant difference between the protein expression levels of the induced and uninduced protein samples, while there is no remarkable difference between the expression levels of His_6-β-Gal and His_6-β-Gal_E537D. The HisProbe blot analysis (Figure 3.2(c)) confirmed the presence of the His_6-tag cloned on the N-terminal of wildtype and E537D β-Gal through chemiluminescence of the HisProbe. The control β-Gal (Lane 2, Figure 3.2(c)) was not detected during the HisProbe blot analysis due to the absence of a His-tag.

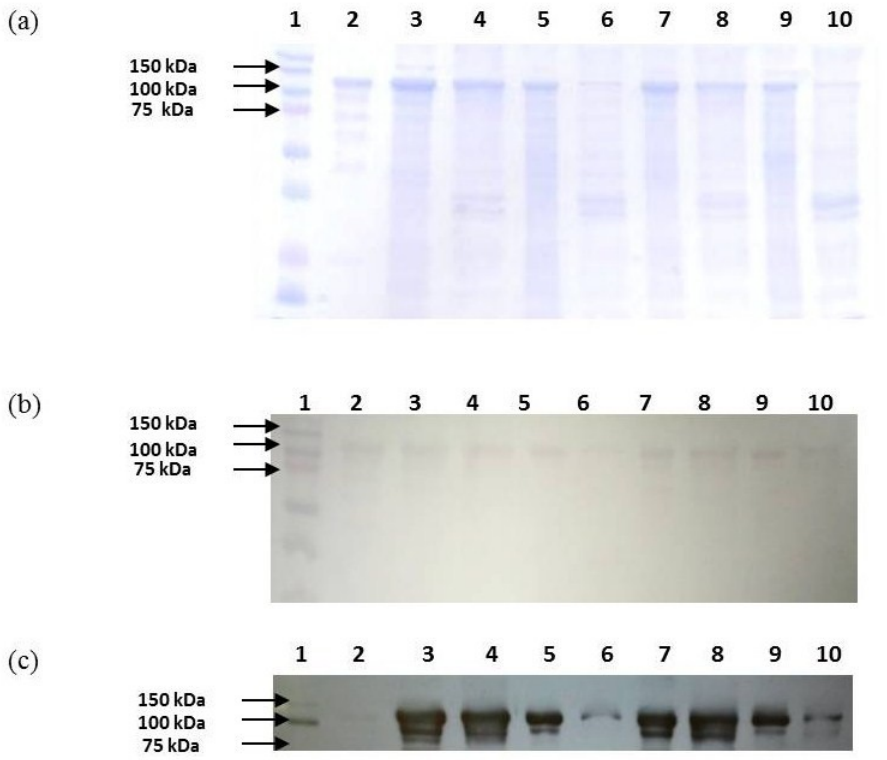


Figure 3.2: (a) SDS-PAGE, (b) Immunoblot and (c) HisProbe blot analyses of two expressed *E. coli Top 10* cell lines, one containing the pTrcHis-*LacZ* plasmid and the other the mutated TrcHis-*LacZ_ E537D* plasmid. The sizes of the molecular weight marker are indicated on the left. Lane 1: Rainbow marker; Lane 2: β-Galactosidase from *Aspergillus oryzae* as a positive control (Sigma-Aldrich) (8 μg); Lane 3: His_6-β-Gal expression induced with IPTG, supernatant sample (10 μg); Lane 4: His_6-β-Gal expression induced with IPTG, pellet sample (5 μg); Lane 5: His_6-β-Gal expression uninduced, supernatant sample (10 μg); Lane 6: His_6-β-Gal expression uninduced, pellet sample (5 μg); Lane 7: His_6-β-Gal_E537D expression induced with IPTG, supernatant sample (10 μg); Lane 8: His_6-β-Gal_E537D expression induced with IPTG, pellet sample (5 μg); Lane 9: His6-β-Gal_E537D expression uninduced, supernatant sample (10 μg); Lane 10: His_6-β-Gal_E537D expression uninduced, pellet sample (5 μg).

3.3.2 IMAC chromatograms of His_6-tagged wildtype and E537D β-Gal

IMAC purification of the wildtype and E537D His_6-β-Gal crude protein mixtures (induced supernatant samples after expression) were performed by eluting the specifically immobilised His_6-β-Gal with a high imidazole concentration from the nickel chelated HiTrap^{TM} column. The resuspended pellet/insoluble fractions after protein expression were not used for protein purification since most of the expressed β-Gal protein was present in the supernatant/soluble fractions (refer to Figure 3.2).

Figure 3.3 illustrates the effectiveness of this IMAC technique in the purification of wildtype and E537D His_6-β-Gal by the clear separation, which is visible in the chromatograms, between the non-specific bound proteins (peak at (a) 7.5 ml, (b) 11.0 ml), other histidine-rich proteins (peak at (a) 20.5 ml, (b) 50.0 ml) and our His_6-tagged protein of interest (peak at (a) 30.0 ml, (b) 79.0 ml). These purification chromatograms (Figure 3.3) for the IMAC purification of wildtype and E537D His_6-β-Gal are similar to those obtained previously [4].

The purified wildtype and E537D His_6-β-Gal fractions were dialyzed against a PBS buffer as described in section 3.2.3.2.

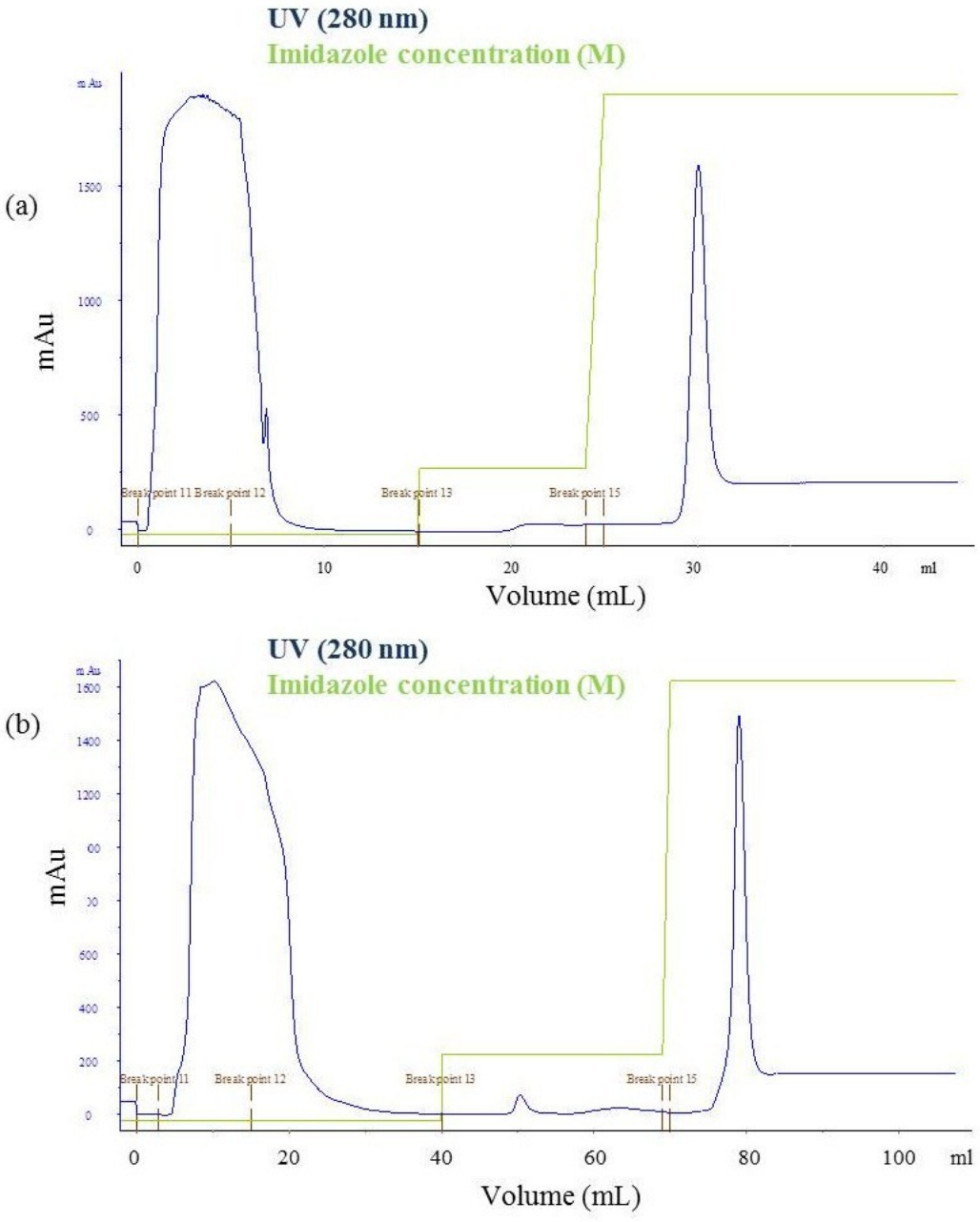


Figure 3.3: Chromatograms obtained with IMAC purification of (a) wildtype and (b) E537D His_6-β-Gal, eluted with an Imidazole gradient of 0 to 0.5 M. The wild-type His_6-β-Gal IMAC purification was conducted using a 1 ml HiTrap^{TM} chelating column while a 5 ml HiTrap^{TM} chelating column was used for the IMAC purification of E537D His_6-β-Gal.

3.3.3 SDS-PAGE and Western blotting verification after protein purification and dialysis

The protein concentrations of the purified wildtype and E537D His_6-β-Gal fractions were estimated to be 0.905 and 0.819 mg/ml, respectively while the total protein yields were 3.6 and 12.3 mg (estimated with BCA protein determination). These concentrations are similar to those previously obtained by Dodd [4].

Although the protein concentrations were very similar, the difference in protein yield is due to the bigger volume of starter lysate (10 ml) used for the purification of E537D His_6-β-Gal with the 5 ml HiTrap^{TM} chelating column that resulted in a larger purified fraction (6 ml) compared to the wildtype. For wildtype His_6-β-Gal only 5 ml starting lysate was used with the 1 ml HiTrap^{TM} chelating column that yielded a purified fraction of only 3 ml.

To verify the presence of β-Gal in the IMAC purified and dialyzed fractions as well as the His_6-tag cloned on the N-terminal of β-Gal; SDS PAGE, immunoblot and HisProbe blot analyses (Figure 3.4) were performed.

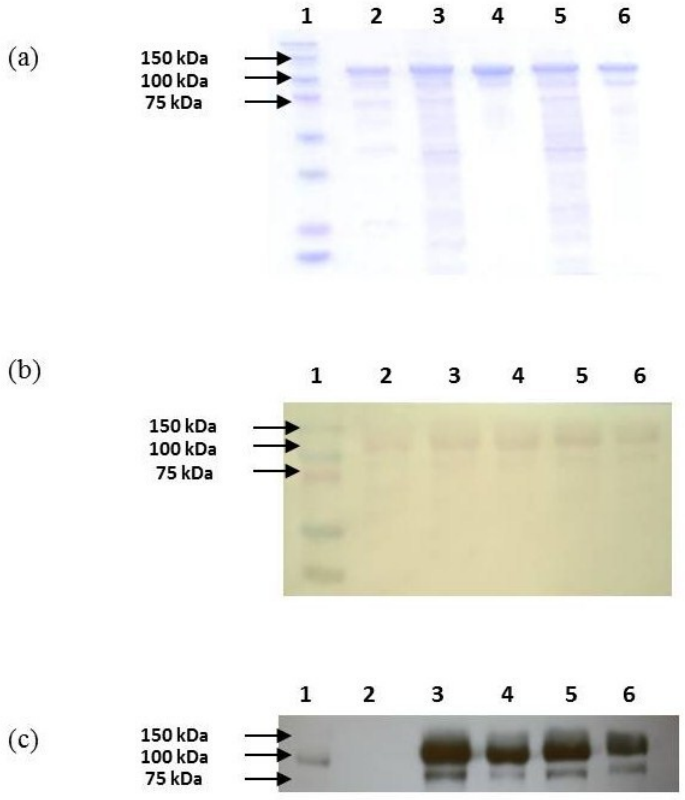


Figure 3.4: (a) SDS-PAGE, (b) Immunoblot and (c) HisProbe blot analyses of IMAC purified wildtype and E537D His_6-β-Gal. The sizes of the molecular weight marker are indicated on the left. Lane 1: Rainbow marker; Lane 2: β-Galactosidase from *Aspergillus oryzae* as a positive control (Sigma-Aldrich) (4 μg); Lane 3: Crude supernatant after expression containing wildtype His_6-β-Gal (8 μg); Lane 4: IMAC purified wildtype His_6-β-Gal (5 μg); Lane 5: Crude supernatant after expression containing E537D His_6-β-Gal (8 μg); Lane 6: IMAC purified E537D His_6-β-Gal (5 μg).

SDS PAGE, immunoblot and HisProbe blot analyses confirmed that His_6-β-Gal (Lane 4, Figure 3.4) and E537D His_6-β-Gal (Lane 6, Figure 3.4) were present and purified from the crude supernatants after expression. This was confirmed by the corresponding sizes of the protein present in the purified fractions (Figure 3.4(a)), the high specificity of the primary antibody during the immunoblot analysis (Figure 3.4(b)) as well as the definite detection of a His-tagged protein at 120 kDa after chemiluminescence of the HisProbe (Figure 3.4(c)).

3.3.4 Michaelis-Menton kinetic properties of wildtype and E537D β-Gal

The effect of substrate concentration on the velocity of the enzymatic hydrolysis reaction of

$$ONPG \xrightarrow{\beta-Galactosidase} o\text{-}nitrophenol(ONP) + Galactose$$

was analyzed at 37 ^{o}C in triplicate with a PowerWave^{TM} HT Microplate Spectrophotometer at 420 nm. ONPG is one of the many different substrates of β-Gal with a β-D-galacto-pyranoside moiety that β-Gal can act upon. The hydrolysis of the terminal β-D-galacto-pyranoside moiety of ONPG by β-Gal results in the formation of the yellow (λ 420 nm) compound ONP [5].

The velocity of this enzymatic reaction increased drastically as the ONPG substrate concentration is increased to 1.6 mM in the presence of the IMAC purified wildtype and E537D His$_6$-β-Gal enzymes (Figure 3.5).

An estimation of the Michaelis constants or K$_m$ and V$_{max}$ was made using the Michaelis Menten equation and nonlinear regression with GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. These results are summarized in Table 3.1.

Table 3.1: GraphPad Prism 5 calculated Michaelis constant (K$_m$) and V$_{max}$ for purified wildtype and E537D His$_6$-β-Gal protein fractions.

Michaelis Menten Best fit values		
	V$_{max}$ (μmol/min/mg)	**K$_m$ (mM)**
Purified Wildtype His$_6$-β-Gal	197.1 ± 4.005	0.21 ± 0.0186
Purified E537D His$_6$-β-Gal	2.87 ± 0.077	0.28 ± 0.0311

The V$_{max}$ for wildtype His$_6$-β-Gal in the hydrolysis of ONPG is 197.1 μmol/min/mg, which is more than 60 times that of E537D His$_6$-β-Gal (Table 3.1). The K$_m$ values, on the other hand, were very similar i.e. 0.21 mM for wildtype His$_6$-β-Gal and 0.28 mM for E537D His$_6$-β-Gal. Through these results, it is, therefore, confirmed that the E537D point mutation reduces

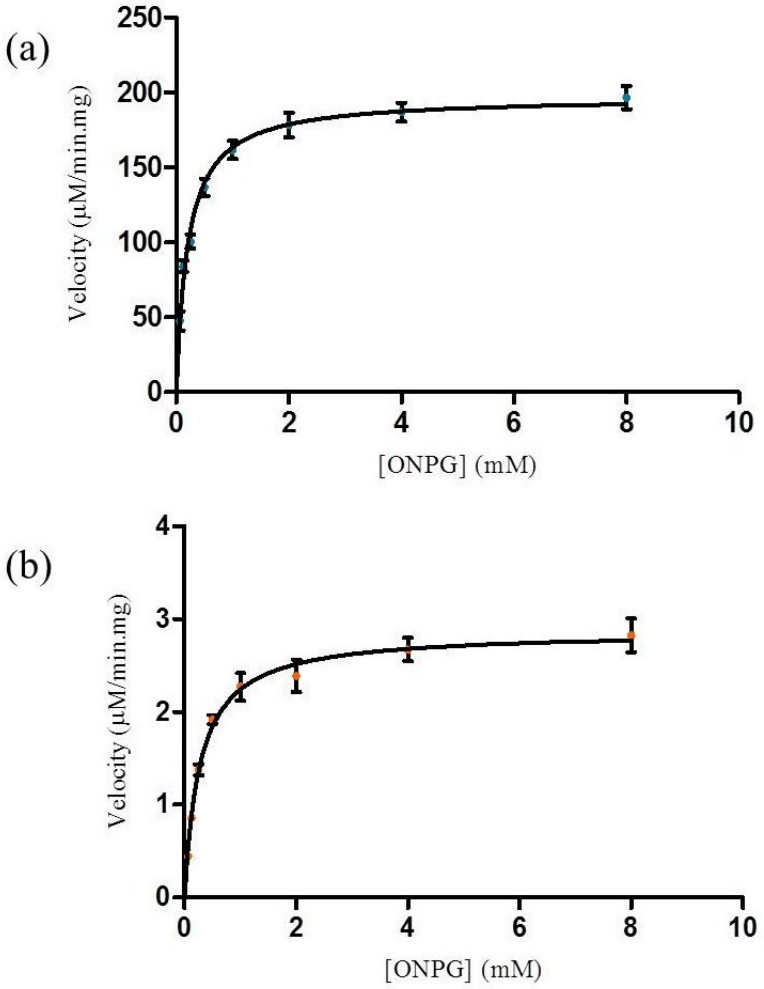


Figure 3.5: Effect of substrate concentration on enzyme velocity. Wildtype and E537D His_6-β-Gal reacted with increasing concentrations of ONPG and the absorbencies were kinetically determined. The average velocity was then plotted against the accompanying ONPG concentration using GraphPad Prism 5 and the Michaelis-Menten equation values for K_m and V_{max} calculated. Error bars indicate standard error of the mean (SEM), n = 3.

the enzymatic activity of β-Gal significantly without significantly altering its substrate binding ability, which is in agreement with previous results from Dodd [4]. The individual values were also similar to those obtained by Dodd [4].

3.4 Conclusion

The isolation of wildtype and E537D His_6-β-Gal via IMAC, produced chromatograms very similar to those obtained by Dodd [4] with a clear separation of His_6-β-Gal from other proteins present in the starting lysate and a high level of purification reproducibility. The purity of the IMAC obtained wildtype and E537D His_6-β-Gal, investigated and verified through SDS PAGE and Werstern blotting, also revealed similar results.

The new microtitre plate assay for β-Gal activity investigations with ONPG revealed similar results to the previously used test tube assay. More specifically, the Michaelis Menten individual constants for both wildtype and E537D His_6-β-Gal was similar to those obtained by Dodd [4]. A V_{max} of 2.87 μmol/min/mg for E537D His_6-β-Gal similar to 2 μmol/min/mg protein previously obtained by Dodd and a K_m of 0.28 mM for E537D His_6-β-Gal similar to 0.27 mM previously obtained by Dodd [4].

Pure and active wildtype as well as E537D His_6-β-Gal have now been obtained, but before these could be covalently attached to functionalized magnetic nanoparticles, the three dimensional structure and topography of β-Gal had to be investigated to determine the most accessible functional groups to be targeted during the subsequent covalent coupling step. This aspect will be discussed in Chapter 4.

Chapter 4

STRUCTURAL INVESTIGATION INTO β-GALACTOSIDASE

4.1 Introduction

The purpose of this investigation was to study the three dimensional structure and topography of β-Gal; the surface accessibility of certain functional groups; and to determine the optimum surface groups that could be targeted for covalent enzyme immobilisation to MNPs.

Due to the large size of the β-Gal tetramer (464 kDa), not many researchers have investigated the specifics regarding ligand binding and functional group accessibility. However, β-Gal has been crystalised by other research groups [93] and we could therefore look at the crystal structure and assess the best method for immobilisation by evaluating the availability of acidic and basic surface residues with available molecular visualization tools. This was not an in depth study since the immobilisation procedure, attempted in Chapter 5, was random and not directed at any specific one amino acid group or peptide chain but rather at basic or acidic residues in general.

After this structural investigation the most appropriate amino acid functional group, according to theoretical calculations, to be targeted during the subsequent coupling step would be selected.

4.2 Materials and Methods

4.2.1 GenBank Database

GenBank (http://www.ncbi.nlm.nih.gov/genbank/) is a NIH genetic sequence database, which provides access to a well organized and annotated collection of all the DNA sequences that are currently available to the public [94]. The sources of β-Gal are endless but we more specifically chose β-Gal from *E. coli* strain K-12 substrain MG1655 since this is the exact strain Dodd [4] used for the PCR amplification and cloning of the *LacZ* gene from *E. coli*.

4.2.2 YASARA 11.3.2 (Yet Another Scientific Artificial Reality Application)

YASARA is a well formulated and user-friendly molecular-graphics, -modeling and -simulation program. YASARA has several unique features with an intuitive user interface, photorealistic graphics and support for affordable autostereoscopic displays, shutter glasses and input devices. The portable vector language (PVL) of YASARA allows for the visualization of even the largest proteins and enables true interactive real-time simulations (http://www.yasara.org/). Here, YASARA was used to calculate and visualize the molecular surface of our protein of interest and to distinguish between the basic and acidic functional groups through differential colouring.

4.2.3 DS Viewer Pro 5.0

DS Viewer Pro 5.0 is a software tool that provides high-quality molecular visualization. This tool provides many different ways of displaying atomic and molecular information on a peptide or protein of interest. DS Viewer Pro 5.0 is user friendly and allowed for the clear display of the sidechains of specified amino acids in the tertiary structure of the enzyme to assess the orientation and accessibility of functional groups.

4.3 Results and Discussion

4.3.1 β-Galactosidase primary amino acid sequence

The primary amino acid sequence of β-Gal from *E. coli* in FASTA format was accessed from GenBank Database (http://www.ncbi.nlm.nih.gov/genbank) using NCBI software (Figure 4.1).

```
>gi|1657540|gb|AAB18068.1| beta-galactosidase
[Escherichia coli str. K-12 substr. MG1655]
MTMITDSLAVVLQRRDWENPGVTQLNRLAAHPPFASWRNSEEARTDRPSQQLRSLNGEWRFAWFPAPEAV
PESWLECDLPEADTVVVPSNWQMHGYDAPIYTNVTYPITVNPPFVPTENPTGCYSLTFNVDESWLQEGQT
RIIFDGVNSAFHLWCNGRWVGYGQDSRLPSEFDLSAFLRAGENRLAVMVLRWSDGSYLEDQDMWRMSGIF
RDVSLLHKPTTQISDFHVATRFNDDFSRAVLEAEVQMCGELRDYLRVTVSLWQGETQVASGTAPFGGEII
DERGGYADRVTLRLNVENPKLWSAEIPNLYRAVVELHTADGTLIEAEACDVGFREVRIENGLLLLNGKPL
LIRGVNRHEHHPLHGQVMDEQTMVQDILLMKQNNFNAVRCSHYPNHPLWYTLCDRYGLYVVDEANIETHG
MVPMNRLTDDPRWLPAMSERVTRMVQRDRNHPSVIIWSLGNESGHGANHDALYRWIKSVDPSRPVQYEGG
GADTTATDIICPMYARVDEDQPFPAVPKWSIKKWLSLPGETRPLILCEYAHAMGNSLGGFAKYWQAFRQY
PRLQGGFVWDWVDQSLIKYDENGNPWSAYGGDFGDTPNDRQFCMNGLVFADRTPHPALTEAKHQQQFFQF
RLSGQTIEVTSEYLFRHSDNELLHWMVALDGKPLASGEVPLDVAPQGKQLIELPELPQPESAGQLWLTVR
VVQPNATAWSEAGHISAWQQWRLAENLSVTLPAASHAIPHLTTSEMDFCIELGNKRWQFNRQSGFLSQMW
IGDKKQLLTPLRDQFTRAPLDNDIGVSEATRIDPNAWVERWKAAGHYQAEAALLQCTADTLADAVLITTA
HAWQHQGKTLFISRKTYRIDGSGQMAITVDVEVASDTPHPARIGLNCQLAQVAERVNWLGLGPQENYPDR
LTAACFDRWDLPLSDMYTPYVFPSENGLRCGTRELNYGPHQWRGDFQFNISRYSQQQLMETSHRHLLHAE
EGTWLNIDGFHMGIGGDDSWSPSVSAEFQLSAGRYHYQLVWCQK
```

Figure 4.1: Primary structure of β-Gal in FASTA format. **E** refers to position 537 in the sequence that was targeted during site-directed mutagenesis by Dodd in 2011 [4].

The primary amino acid sequence of 1023 amino acid residues were used to calculate the amino acid composition and molecular percentages with DS

ViewerPro 5.0 software. Table 4.1 contains the amino acid composition of one subunit of β-Gal.

Table 4.1: Amino acid composition of β-Gal (gi|1657540) calculated using DS ViewerPro software.

Amino Acid	Number	Mol %
Asp D	64	6.25
Glu E	62	6.05
Lys K	20	1.95
Arg R	66	6.45
His H	34	3.32
Cys C	16	1.56
Met M	24	2.34
Leu L	96	9.38
Ile I	39	3.81
Val V	64	6.25
Phe F	38	3.71
Pro P	62	6.05
Ala A	77	7.52
Asn N	47	4.59
Gln Q	58	5.66
Gly G	71	6.93
Ser S	60	5.86
Thr T	56	5.47
Trp W	39	3.81
Tyr Y	31	3.03

It was calculated from Table 4.1 that there were approximately equal amounts of acidic (126) and basic (120) residues. The sum of the molecular percentages of the acidic residues (Asp and Glu) was 12.3 % and that of the basic residues (Lys, Arg and His) was 11.72 %. It can therefore be assumed that these functional group containing amino acid residues are similar with regards to abundance in the homotetrameric form of the enzyme. β-Gal also contains a relatively small amount (3.90 %) of thiol group containing amino acid residues (Cys and Met) that could also potentially be targeted during enzyme immobilisation. The neutral amino acid residues (Leu, Ile, Val, Phe, Pro and Ala) are the most predominant (36.72 %) in this enzyme of interest.

4.3.2 Investigation into a possible functional group as target for the covalent immobilisation of β-Gal

4.3.2.1 Secondary structure representation

The secondary structure motifs of β-Gal from *E. coli* was visualized within the homotetrameric tertiary structure of the enzyme using YASARA software (DOI:10.2210/pdb1hn1/pdb). In Figure 4.2 the β-sheets are visible in red and the α-helixes in blue. The four active sites are surrounded by several β-sheets and α-helixes, which may be responsible for the conserved deep pocket conformation of the β-Gal active site.

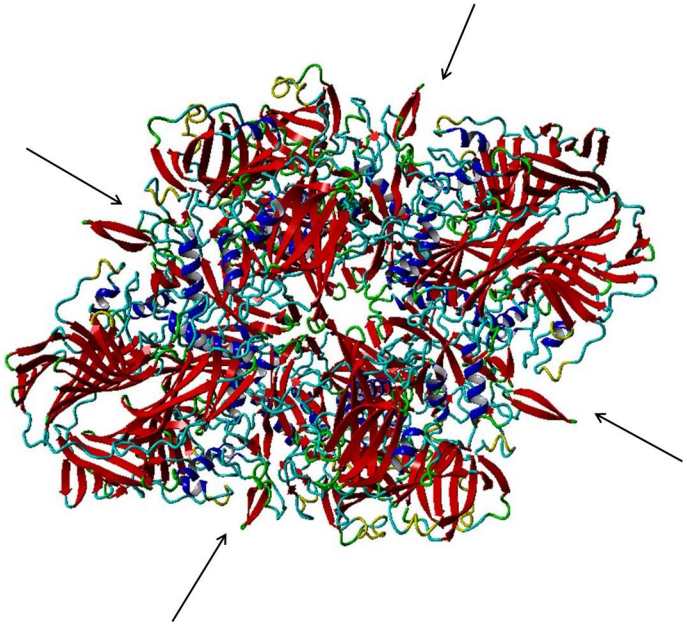


Figure 4.2: Secondary structure motifs of β-Gal from *E. coli* strain K-12 substrain MG1655 differentially coloured. β-Sheets visible in red, α-helixes in blue, random coils (cyan) and turns (yellow). Arrows indicate 4 active sites present in the homotetrameric tertiary structure.

4.3.2.2 Tertiary structure

By using YASARA and DS ViewerPro the crystal three dimensional structure of β-Gal from *E. coli* was visualized (Figure 4.3). In this figure, both the ball and stick style (Figure 4.3(a)) and the ribbon style (Figure 4.3(b)) visualization were used to represent the three dimensional structure of β-Gal.

As previously mentioned, β-Gal is a homotetramer with monomers of 116 kDa each. Three amino acids, Met502, Glu537 and Glu461, which have been shown to be involved in the catalytic activity [19] are indicated in Figure 4.3(b).

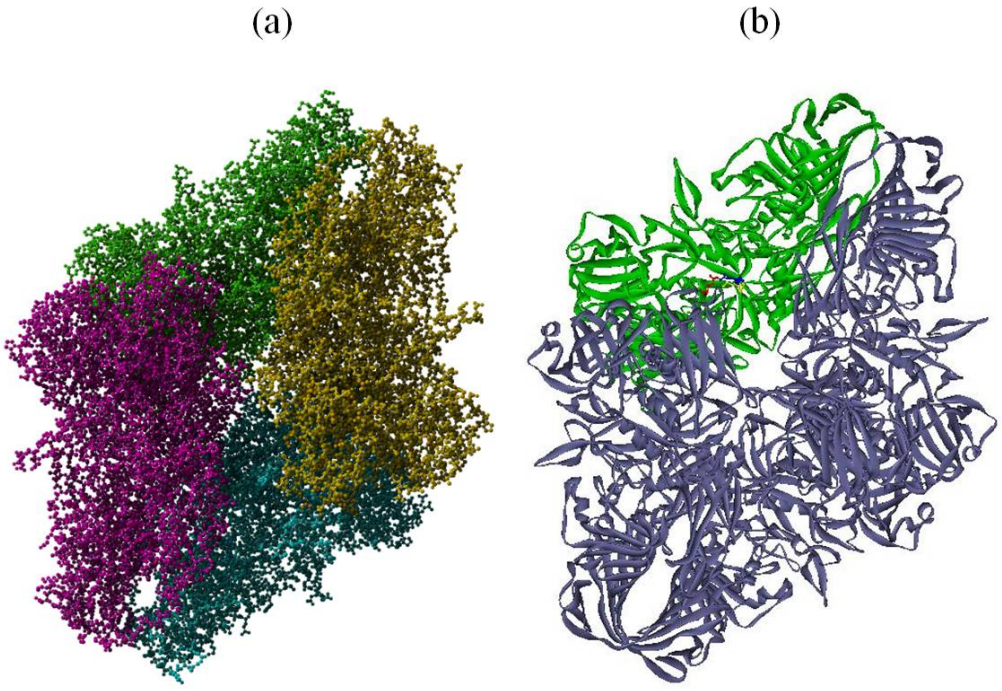


Figure 4.3: Three dimensional structure of β-Gal. (a) Four subunits of homotetramer differentially coloured in ball and stick style. (b) One subunit of the homotetramer indicated in green with the primary structure and orientation of the amino acid residues located to a single active site indicated in different colours i.e. Glu461 (Red), Met502 (Yellow) and Glu537 (Blue).

4.3.2.3 Visualization of functional group orientation

To determine the optimum functional group, with regards to abundance and accessibility, to be targeted for potential covalent immobilisation of this complex enzyme, a more in-depth investigation into the accessibility and orientation of the functional groups of the basic and acidic amino acid residues was necessary. Using DS ViewerPro 5.0 all of these could be calculated or visualized.

Figure 4.4 depicts the accessibility of the acidic amino acid (Asp and Glu) residues of β-Gal. It seems as though the carboxylic groups of the acidic residues are directed more towards the inside of the homotetramer. On the other hand, it appears as though the amino groups of the basic residues (Lys, Arg and His) (Figure 4.5) are directed more towards the outside of the enzyme. However, these predictions are insufficient since it only relies on side chain orientation and cannot be used as the only information on deciding which functional group to be targeted for potential peptide bond formation for the ultimate covalent immobilisation of β-Gal.

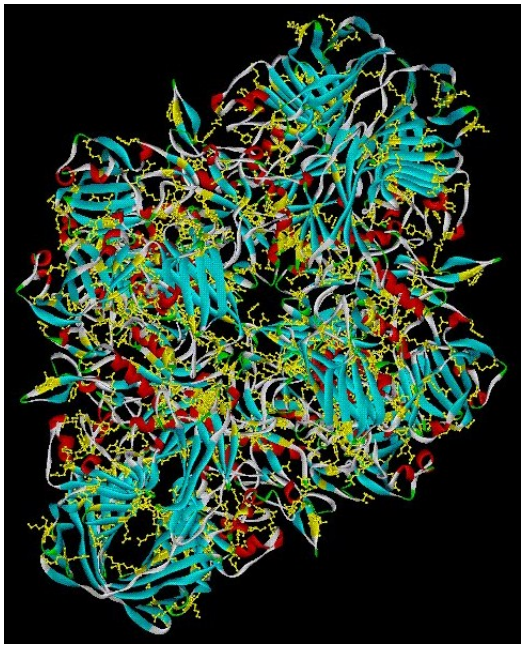


Figure 4.4: Accessibility of functional groups of acidic amino acid (Asp and Glu) residues of β-Gal, indicated in yellow, as visualized with DS ViewerPro software.

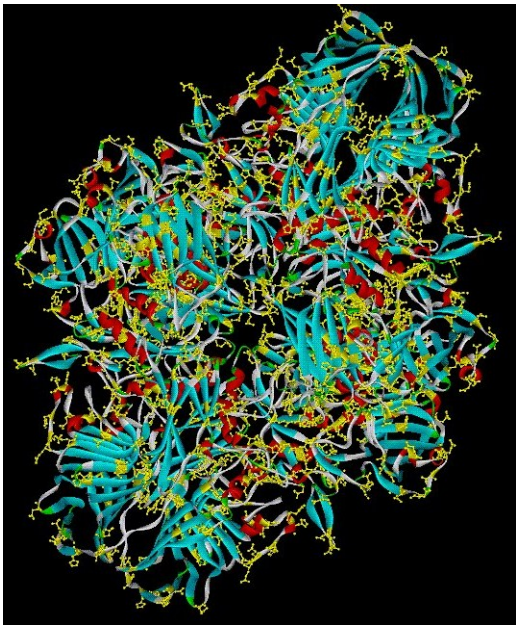


Figure 4.5: Accessibility of functional groups of basic amino acid (Lys, Arg and His) residues of β-Gal, indicated in yellow, as visualized with DS ViewerPro software.

YASARA software was then used to calculate and visualize the molecular surface of β-Gal to support the previous accessibility investigation and to also incorporate the area and volume taken up by each sidechain. Here, Figure 4.6, the molecular surface of the functional groups were differentially coloured to visualize the surface accessibility of these groups. The carboxylic functional groups (acidic residues) were coloured in red while the amine functional groups (basic residues) were coloured in green.

From Figure 4.6 it appears as though both these functional groups are equally accessible when the area and volume of the sidechains are also taken into consideration. It was, therefore, decided that any one of these groups could be targeted for the potential covalent immobilisation of β-Gal.

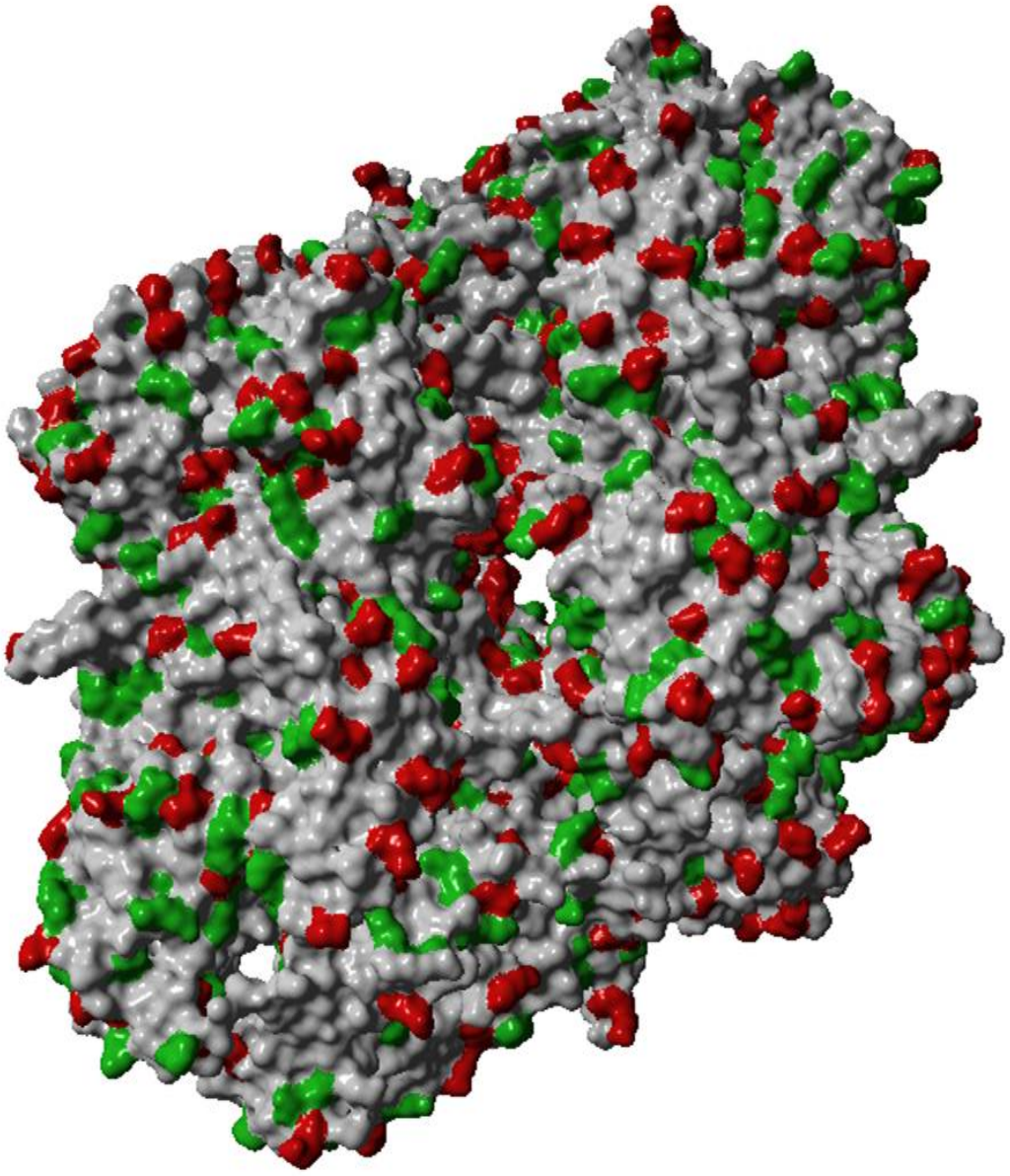
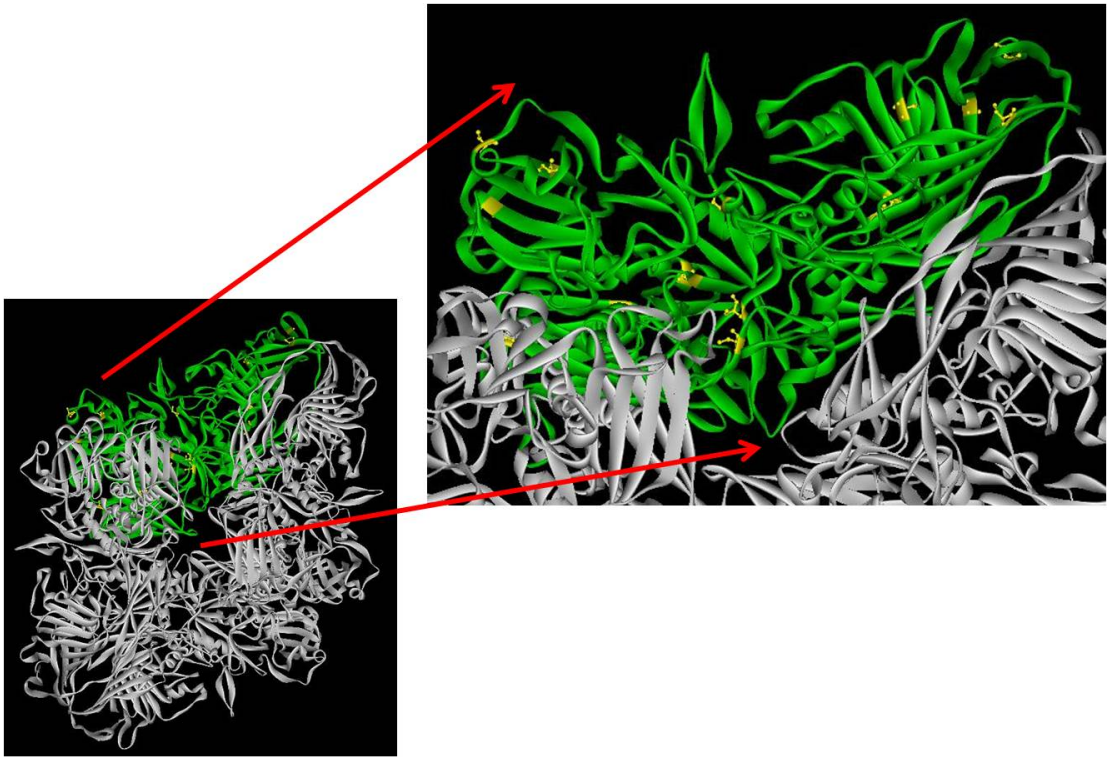


Figure 4.6: Molecular surface accessibility of functional groups of acidic and basic amino acid residues of β-Gal. Acidic amino acid residues indicated in red and basic in green, as visualized with YASARA software.

The thiol group of Cysteine residues is another possible target for covalent enzyme immobilisation through the formation of a disulphide bridge. As depicted in Figure 4.7, the functional thiol group of the cysteine residues is protected in the tertiary structure of β-Gal. The cysteine residues of β-Gal would therefore not be targeted during potential immobilisation strategies in the subsequent chapters.



(a) (b)

Figure 4.7: Accessibility of functional groups of cysteine amino acid residues of β-Gal, indicated in yellow, as visualized with DS ViewerPro software.(a) β-Gal homotetramer visible with cysteine residues (yellow) indicated in one (green) of the four subunits. (b) Enlarged version of (a) with the cysteine residues more visible.

4.4 Conclusion

From the three dimensional structural investigation of β-Gal it appears as though the four active sites in the homotetrameric form of the enzyme are equally accessible to the ligand. The abundance as well as the molecular surface accessibility investigation of the functional groups of the basic and acidic amino acid residues indicated both of these to be equally abundant and accessible. It is due to this finding that it was decided that both the -COOH and -NH_3 could be targeted during the covalent coupling of the protein to the MNPs. Furthermore, this coupling of the protein to the MNPs would then be random since either the carboxylic groups or amine groups as a whole would be targeted instead of directing the coupling towards a single site.

Chapter 5

A COMPARISON BETWEEN EDC AND GLUTARALDEHYDE FOR RANDOM COVALENT IMMOBILISATION OF E537D β-GAL

5.1 Introduction

The random covalent immobilisation of E537D His_6-β-Gal to MNPs was performed on commercially available surface activated MNPs - fluidMAG-Amine (Figure 5.1), which has a superparamagnetic magnetite core coated with a amino silane polymer matrix with surface accessible amine functional groups. According to Van den Berg *et al.* [95], controlled covalent immobilisation is highly desirable since the binding between the protein and surface is strong and permanent. The immobilisation strategy employed aimed to target the surface accessible amino acid groups of β-Gal to be covalently linked to the amine groups on the surface activated fluidMAG-Amine particles. Two differ-

ent covalent immobilisation strategies were investigated and compared to yield these functional immobilised enzyme particles.

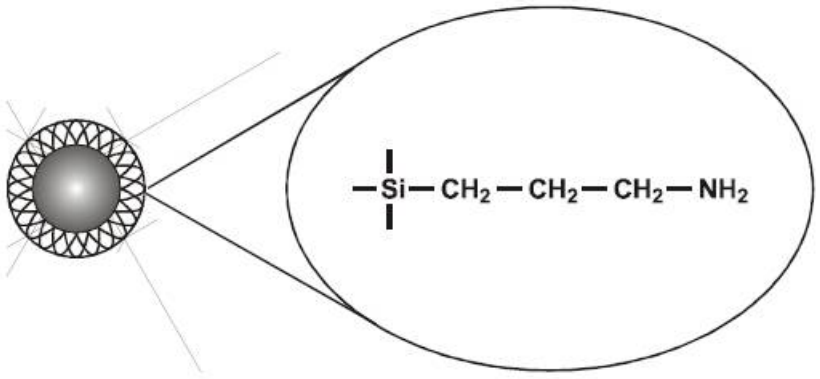


Figure 5.1: Chemicell fluidMAG-Amine product representation (Article no. 4121, 200 nm).

N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (Figure 5.2) is a coupling agent widely used for the covalent coupling of proteins to solid supports [63]. The mechanism of action of EDC relies on the fact that EDC activates carboxylic groups by forming a reactive intermediate product called O-acylisourea. This reactive intermediate, containing a good leaving group, catalyzes the amide bond formation between the activated carboxylic group and a primary amine group. Isourea, with a yellowish colour, is formed as a by-product and can be easily removed through several wash steps with buffer or water [96]. EDC is also known as a "zero-length" cross-linking agent since the amide bond is formed without leaving a spacer molecule [97].

Although EDC has previously been used by a number of researchers, for covalent coupling, the results of a number of such studies lacked the controls required to eliminate the possibility of protein adsorption. These discrepancies brought into question the feasibility of applying EDC in protein immobilisation studies. However, manufacturer's instructions (fluidMAG-Amine, Chemicell) recommended using EDC and it is for this reason that the EDC activation of the carboxylic groups present in the protein of interest was performed in

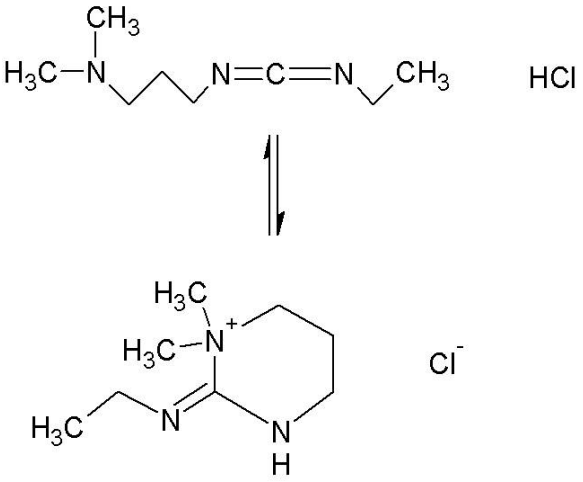


Figure 5.2: Chemical structure of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC).

this study. The extremely reactive intermediate, O-acylisourea, was used to catalyze the coupling of the surface accessible carboxylic groups, present on the protein, to the primary functional amine-groups on the surface of the fluidMAG-Amine particles through the formation of amide bonds.

Glutaraldehyde (Figure 5.3) is another well-known and very effective protein cross-linking agent, used to yield functional immobilised enzyme particles. Glutaraldehyde is very reactive towards proteins, especially in its application in the production of insoluble enzymes [98]. It can react with several functional groups on proteins, such as amine, phenol, thiol and imidazole, as nucleophiles are the most reactive amino acid side-chains [98]. In addition, various aldehyde reactivities have been reported with lysine, tryptophane, tyrosine, phenylalanine, cysteine, histidine, proline, glycine, glycylglycine, serine and arginine [98]. It is, therefore, possible to rank the reactivity of aldehydes towards reactive moieties of amino acids in increasing order of reactivity as follows: hydroxyl groups, secondary amino, guanidinyl, α-amino and ϵ-amino as the most reactive towards aldehydes [98].

Aldehydes, like glutaraldehyde, are usually expected to form Schiff bases upon nucleophilic attack by the amine groups of residues in the protein of interest [99]. These Schiff bases are, however, unstable under acidic conditions

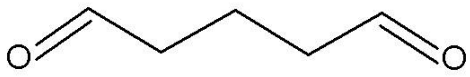


Figure 5.3: Chemical structure of monomeric glutaraldehyde.

and usually tend to break down into the separate aldehyde and amine if not reduced by a reducing agent like $NaBH_4$, which converts the unstable Schiff base to a stable secondary amine [100]. In contrast, it appears as though the reaction of glutaraldehyde with an amino group is not likely to follow the above mentioned mechanism but is instead exceptionally stable under extreme pHs and temperatures [98; 100]. It is for this reason that the simple mechanism, where both ends of monomeric glutaraldehyde reacts with amino groups through a Schiff base, has been ruled out and that cross-linking of proteins with glutaraldehyde does not require a reducing agent [98]. Refer to [98] for a more detailed description of glutaraldehyde behaviour in aqueous solutions with emphasis on reactions with proteins and enzyme cross-linking.

It is, thus, clear from the above that apart from all the available literature on glutaraldehyde cross-linking and proposed molecular reactive forms, no single mechanism seems to be responsible for glutaraldehyde protein cross-linking. Evidence suggests that the multi-component nature of glutaraldehyde presents at least 13 different forms depending on reaction conditions [98]. Therefore conditions, including pH, ionic strength, temperature, enzyme and reagent concentrations, and the reaction time all need to be optimized for every protein of interest. Some guidelines regarding the pH, temperature and reaction times are provided by Migneault *et al.* [98]. These suggestions include the following: maintain the pH of the solution close to the isoelectric point (pI) of the protein [101], reaction temperatures of 4 ^{o}C require longer reaction times (6–18 h) [102]; while reactions at ambient temperatures are completed within 4 h [103].

The main difference between EDC and glutararaldehyde, expected to in-

fluence the amount of protein that can be coupled to the MNPs as well as the activity of the immobilised enzyme, is the "zero-length" cross-linking of EDC compared to the incorporation of a spacer with glutaraldehyde cross-linking. This spacer incorporated with glutaraldehyde cross-linking might allow more protein to be coupled to the MNP surface as well as limit steric hindrances [61].

5.2 Materials and Methods

5.2.1 Reagents and chemicals

Crude extract of E537D His_6-β-Gal overexpressed in *E. coli* (*Top 10 One Shot®*) was produced and purified through IMAC as previously described (section 3.2.2.1 and 3.2.3.1). FluidMAG-Amine (Article no. 4121, 200 nm diameter), amine surface activated magnetic nanoparticles, were ordered from *Chemicell* (Berlin, Germany). Commercial grade N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and glutaraldehyde solution 50 wt. % in water were purchased from *Sigma-Aldrich Chemical Co.*(Germany) and all chemicals used for the preparation of buffers and solutions were of analytical grade or higher and purchased from *Merck*, *Sigma-Aldrich Chemical Co.* or *Bio-Rad*. An Ultra Pure Milli-Q water system was used to produce reagent grade water used in all buffers and experiments.

5.2.2 Covalent coupling of E537D β-Gal to fluidMAG-Amine by EDC

EDC with a M_w=191.7 g/mol, is a water soluble, white to off-white coloured powder. It is stable but sensitive to moisture as well as being incompatible with strong oxidizing agents and strong acids.

IMAC purified protein was dialyzed against a PBS buffer, pH 7.4 (refer to

section 3.2.3.2). FluidMAG-Amine particles (1 mg (40 μl) of the 25 mg/ml stock suspension) were added to clean microtubes where each microtube represented a potential immobilisation reaction between the protein of interest and 1 mg of particles. The EDC solution (freshly prepared immediately before use by dissolving EDC (5 mg) in sterile deionised water (0.075 ml)) (15 μl) was rapidly mixed into each reaction tube by means of a vortex (only the necessary volume of EDC was prepared each time in the above ratios). E537D β-Gal was added to the fluidMAG-Amine and EDC mixture in the tubes and gently mixed on a Labroller for 2 hours at room temperature. After the two hour incubation period, the particles were separated from the slightly yellow supernatant through magnetic decantation and the supernatant carefully removed with a pipette (labeled: supernatant). The particles were then washed three times with PBS buffer (100 μl, pH 7.4) and the supernatant again separated from the particles through magnetic decantation (labeled: wash steps 1–3). Finally the particles were resuspended in PBS buffer (100 μl, pH 7.4) and stored at 4 ^{o}C for further analysis. All supernatant and wash step samples were analyzed to calculate the amount of E537D β-Gal immobilised to 1 mg of particles (refer to section 5.2.4).

The amount of E537D β-Gal immobilised to the fluidMAG-Amine was optimized experimentally. Different amounts (20, 40, 60, 80, 90, 100, 120, 130, 140, 150 and 160 μg) of a 1 mg/ml E537D β-Gal solution were added to the fluidMAG-Amine in triplicate and all supernatant and wash step samples analyzed (refer to section 5.2.4). The resulting data were used to draw a saturation curve and determine the optimum amount of protein (inμg) that could be immobilised to 1 mg of fluidMAG-Amine as represented by GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

5.2.3 Covalent cross-linking of E537D β-Gal to fluidMAG-Amine with glutaraldehyde

Glutaraldehyde is a linear, 5-carbon dialdehyde that is clear and colourless to pale-straw in colour. It is a pungent oily liquid that is soluble in alcohol, water and organic solvents.

The covalent immobilisation of E537D β-Gal to fluidMAG-Amine via cross-linking with glutaraldehyde was performed as described by Rossi *et al.* [104] and slightly adapted for the purpose of this study. It must be noted that Rossi *et al.* made use of glutaraldehyde to immobilise glucose oxidase, and not β-Gal, to amine functionalized MNPs.

The IMAC purified protein was dialyzed against a PBS buffer, pH 7.4 (refer section 3.2.3.2). FluidMAG-Amine particles (1 mg) were re-dispersed in 10 % glutaraldehyde PBS solution, pH 7.4 (400 μl), by sonication in microtubes and subsequent gentle mixing on a Labroller for 1 hour at room temperature. Hereafter, the particles were separated through magnetic decantation, washed twice with PBS buffer (100 μl, pH 7.4) and finally re-suspended in PBS buffer (20 μl, pH 7.4). E537D β-Gal was added to the glutaraldehyde functionalized particles and gently mixed for four hours at ambient temperature. The particles were subsequently separated through magnetic decantation, washed three times in PBS buffer, pH 7.4 and once in PBS buffer, pH 6.5 before resuspension in PBS buffer (100 μl, pH 7.4). The supernatant, and wash step samples, were kept for further analysis (refer to section 5.2.4). The PBS re-suspended particle samples were stored at 4 ^{o}C until further analysis.

As for the EDC coupling method for protein immobilisation, the amount of E537D β-Gal immobilised to the fluidMAG-Amine was also optimized for the glutaraldehyde cross-linking method. Apart from this, the solution pH, reaction temperature and time were also optimized according to literature [98].

It was suggested by Jansen *et al.* [101] that the pH of the solution should be

as close as possible to the pI of the protein of interest, which is 5.3 for β-Gal, since protein charge plays an important role in intermolecular cross-linking that is required for insolubilization. On the other hand, the optimal reactivity pH for aqueous glutaraldehyde should also be taken into consideration. Most glutaraldehyde immobilisation experiments are currently conducted at a neutral or slightly alkaline pH. It was therefore decided to keep the reaction pH at the PBS buffer pH of 7.4 since β-Gal is stable and active over a wide pH range (5 - 8.5).

Tween-20 was included during the immobilisation strategies, as advised by Van den Berg *et al.* [95], since they found that the coincubation of protein and Tween during the incubation step for immobilisation effectively inhibited hydrophobic association of the protein with surfaces. Tween 20, to a final concentration of 0.2 %, was therefore included in protein and fluidMAG-Amine incubations during the coupling step.

The glutaraldehyde coupling step was ultimately performed at ambient temperature for four hours as current practice for glutaraldehyde immobilisation studies, suggested by Krogh *et al.* [103], since β-Gal is not a labile molecule. The incubation time and temperature were also investigated experimentally and the results indicated that the reaction was complete within four hours at ambient temperature, whereas the reaction took significantly longer to complete at 4 ^{o}C. By performing the coupling step as described above, the immobilisation reaction was allowed to occur under physiological conditions (pH 7.4 and 25 ^{o}C) as previously mentioned in section 2.4.4 (Camarero [61]).

5.2.4 Determination of the amount of E537D β-Gal protein immobilised to 1 mg fluidMAG-Amine

BCA protein determination assays (refer to section 3.2.2.2) were used to determine the maximum amount of E537D β-Gal immobilised to 1 mg of fluidMAG-Amine through EDC coupling and glutaraldehyde cross-linking.

During the course of the optimization experiment, the temperature (ambient), pH (7.4), incubation time (2 hours for EDC method and 4 hours for glutaraldehyde method), reaction volume (215 μl for the EDC method and 200 μl for the glutaraldehyde method) and amount of fluidMAG-Amine (1 mg) were kept constant while the concentration of E537D β-Gal was varied between 0 and 0.8 mg/ml. The reason for the slightly bigger reaction volume for the EDC immobilisation samples is because the EDC solution was part of the final reaction volume (refer to section 5.2.2). By comparing the protein estimation of the supernatant before and after immobilisation (including the wash step samples), the difference in enzyme concentration indicated the maximum amount (in μg) of E537D β-Gal immobilised to 1 mg fluidMAG-Amine. This method of measuring the loss of protein in solution is a well-documented indirect method to determine the amount of immobilised protein [63].

5.2.5 Adsorption of protein and removal by detergent

In the desorption experiment, to remove all protein that was not covalently linked to the fluidMAG-Amine, the EDC and glutaraldehyde coupled E537D β-Gal particles as well as a control sample without a coupling agent, were resuspended in a 2 % SDS-PBS buffer, pH 7.4, solution at 50 ^{o}C for 1 hour with mixing as previously described by Van den Berg *et al.* [95]. The particles were subsequently removed from the SDS-PBS solution by magnetic decantation and the supernatant stored at 4 ^{o}C. The particles were washed several times with sterile deionized water, lyophilized and analyzed by Attenuated Total Reflectance Fourier Transform Infrared vibrational spectroscopy (ATR-FTIR) to determine non-specific as well as specific covalent binding of E537D β-Gal. The supernatant samples, obtained from the SDS wash step, were also analyzed by a protein concentration determination to estimate the amount (in μg) of E537D β-Gal that was originally physically adsorbed to the fluidMAG-Amine surface and was effectively desorbed during the detergent wash step.

5.2.6 Verification of covalent immobilisation through Attenuated Total Reflectance Fourier Transform Infrared vibrational spectroscopy (ATR-FTIR)

Samples of the fluidMAG-Amine coupled E537D β-Gal (via EDC (section 5.2.2) or glutaraldehyde (section 5.2.3) immobilisation) as well as two control samples, one containing pure fluidMAG-Amine and the other containing fluidMAG-Amine mixed with E537D β-Gal in the absence of coupling or cross-linking agents, were prepared together with the SDS washed samples (section 5.2.5) for ATR-FTIR analysis.

Sample preparation consisted of the washing and resuspension of samples in sterile deionised water followed by overnight lyophilisation. The resulting powdered samples were used for the analysis.

ATR-FTIR analysis was performed by means of OMNIC software on a Thermo Scientific Nicolet iS10 SMART iTR at the Department of Polymer Science, Stellenbosch University. The absorbance spectra of all the samples were collected and compared to the control, pure fluidMAG-Amine.

5.2.7 Verification of fluidMAG-Amine surface coating with E537D β-Gal using Transmission Electron Microscopy (TEM)

TEM was conducted at the University of Cape Town by Mr. Franscious Cummings. The light microscope used was a TECNAI F20 Field Emission gun transmission electron microscope, operated at 200 kV with the collection of bright field images. Nitrogen gas was used to cool the TEM system down before sample analysis.

Specimen preparation consisted of the washing (three times with 100 μl) and resuspension (40 μl) of the uncoated and protein coated fluidMAG-Amine samples in sterile deionised water. The samples were then prepared by diluting

3 μl of the suspended particles in 20 μl of deionised water. The suspensions were then sonicated for 10 minutes to improve the dispersion of the specimen within the solution. After sonication one drop of the uncoated fluidMAG-Amine particles was placed on a glow-discharged carbon coated copper grid (300 mesh). The glow-discharge process renders the grids hydrophilic, which enables the water-dispersed samples to spread out over the grid. The uncoated fluidMAG-Amine sample was then allowed to dry under a 240 W light source.

The protein coated samples, i.e. E537D β-Gal coated fluidMAG-Amine via EDC and via glutaraldehyde cross-linking, were stained using uranyl acetate. One drop of the sample was placed on a glow-discharged carbon coated copper grid for 30 seconds, whereafter excess sample was blotted off using filter paper. The grid was subsequently placed on a drop of uranyl acetate (2 wt% in deionised water), blotted dry and this last staining step repeated, hereafter the grid was allowed to dry in air until analysis.

5.2.8 Verification of fluidMAG-Amine surface coating with E537D β-Gal using Scanning Electron Microscopy (SEM)

SEM analysis was conducted at the Electron Microscope Unit of the University of Cape Town by Ms. Miranda Waldron in order to study the surface topography and composition of the MNPs. The microscope used was a Nova NanoSEM 230 and was operated at a high voltage of 5.00 kV and a chamber pressure of 1.00 mbar.

Specimen preparation consisted of the overnight lyophilization of the uncoated and protein coated fluidMAG-Amine samples. The powdered samples were then located to a SEM stub that was coated with a thin layer of homemade carbon glue, and numbered. After sample application the excess powder was blown off and the specimens coated with gold for 30 minutes. Hereafter the

specimens were visualized by SEM.

5.3 Results and Discussion

5.3.1 BCA protein determination of supernatant samples to verify protein immobilisation

As expected, the BCA protein determination of the supernatant samples, before and after E537D β-Gal immobilisation, to fluidMAG-Amine via the two strategies, yielded a lower protein concentration after immobilisation than before immobilisation. This was indicative of protein immobilisation to fluidMAG-Amine, but did not suggest or indicate covalent attachment. The possible covalent attachment needed to be investigated and verified separately.

From Tables 5.1 and 5.2 it is evident that considerably more protein remained in the supernatant after immobilisation as the protein concentration of the protein, incubated with the particles, was increased (refer to section 5.2.4 for increased protein concentrations). This phenomenon was more pronounced for the supernatant samples obtained from the EDC method (Table 5.1, 61 % of 0.8 mg/ml protein remained in the supernatant) than for the samples obtained from the glutaraldehyde method (Table 5.2, 45 % of 0.8 mg/ml protein remained in the supernatant). However, control samples with fluidMAG-Amine and E537D β-Gal, where the cross-linking and coupling agents were omitted, also indicated a decreased protein concentration after the immobilisation incubation step (data not shown). This suggested the physical adsorption of protein to the fluidMAG-Amine. In order to distinguish between the physically adsorbed and covalently attached protein, as well as to verify the covalent immobilisation of the protein of interest, a detergent wash step was incorporated after the normal coupling step to remove any physi-sorption of protein (refer to section 5.2.5).

Table 5.1: Protein concentration estimations of E537D β-Gal supernatant samples before and after immobilisation via EDC coupling method as determined by the Pierce BCA method.

Volume of 1 mg/ml E537D β-Gal added (ml)	**Duplicate Supernatant protein concentration before immobilisation (mg/ml)**		**Duplicate Supernatant protein concentration after immobilisation (mg/ml)**		**Saturation curve data**		
					[a]Protein bound to fluidMAG-Amine (μg E537D β-Gal/ mg fluidMAG-Amine)		Protein concentration added (mg/ml)
0.000	0.000	0.000	0.000	0.000	0.00	0.00	0.000
0.020	0.093	0.093	0.000	0.000	20.00	20.00	0.093
0.040	0.186	0.186	0.000	0.000	40.00	40.00	0.186
0.060	0.279	0.279	0.060	0.061	47.62	47.41	0.279
0.080	0.372	0.372	0.132	0.134	52.96	52.55	0.372
0.090	0.419	0.419	0.172	0.166	54.70	55.94	0.419
0.100	0.465	0.465	0.211	0.212	56.66	56.45	0.465
0.120	0.558	0.558	0.275	0.283	63.65	62.00	0.558
0.130	0.605	0.605	0.320	0.313	64.36	65.81	0.605
0.140	0.651	0.651	0.355	0.360	67.14	66.11	0.651
0.150	0.698	0.698	0.400	0.399	68.06	68.26	0.698
0.160	0.744	0.744	0.457	0.433	66.29	71.25	0.744

[a]Values calculated by multiplying the volume of the supernatant after immobilisation (205 μl) with the supernatant protein concentration after immobilisation and subtracting this total (not bound to fluidMAG-Amine) from the amount of protein originally incubated with fluidMAG-Amine (Volume of 1 mg/mL multiplied by 1 to obtain mg E537D β-Gal added, i.e. Protein bound to fluidMAG-Amine$_{\mu g}$ = Protein added $_{\mu g}$ - ($V_{supernatant_{ml}} \times C_{[supernatantafter]_{mg/ml}} \times 1000$)

Table 5.2: Protein concentration estimations of E537D β-Gal supernatant samples before and after immobilisation via glutaraldehyde cross-linking method as determined by the Pierce BCA method.

Volume of 1 mg/ml E537D β-Gal added (ml)	**Duplicate Supernatant protein concentration before immobilisation (mg/ml)**		**Duplicate Supernatant protein concentration after immobilisation (mg/ml)**		**Saturation curve data**		
					[a]Protein bound to fluidMAG-Amine (μg E537D β-Gal/ mg fluidMAG-Amine)		Protein concentration added (mg/ml)
0.000	0.000	0.000	0.000	0.000	0.00	0.00	0.000
0.020	0.100	0.100	0.039	0.034	12.18	13.16	0.100
0.040	0.200	0.200	0.085	0.094	23.39	21.61	0.200
0.060	0.300	0.300	0.128	0.135	33.90	32.60	0.300
0.080	0.400	0.400	0.181	0.193	44.34	42.06	0.400
0.090	0.450	0.450	0.195	0.200	50.71	49.74	0.450
0.100	0.500	0.500	0.230	0.238	54.05	52.58	0.500
0.120	0.600	0.600	0.270	0.268	67.42	67.75	0.600
0.130	0.650	0.650	0.301	0.290	70.90	73.01	0.650
0.140	0.700	0.700	0.316	0.316	77.62	77.62	0.700
0.150	0.750	0.750	0.337	0.334	83.04	83.69	0.750
0.160	0.800	0.800	0.367	0.364	87.01	87.66	0.800

[a]Values calculated by multiplying the volume of the supernatant after immobilisation (190 μl) with the supernatant protein concentration after immobilisation and subtracting this total (not bound to fluidMAG-Amine) from the amount of protein originally incubated with fluidMAG-Amine (Volume of 1 mg/mL multiplied by 1 to obtain mg E537D β-Gal added, i.e. Protein bound to fluidMAG-Amine$_{\mu g}$ = Protein added $_{\mu g}$ - ($V_{supernatant_{ml}} \times C_{[supernatantafter]_m g/ml} \times 1000$)

5.3.2 Verification of desorption of adsorbed protein

A SDS wash step was employed after the immobilisation and PBS buffer wash steps to remove non covalently bound E537D β-Gal from the fluidMAG-Amine. These SDS-PBS wash solutions were kept for a protein determination to estimate the amount of protein removed during the detergent wash from the fluidMAG-Amine.

Observations made during the immobilisation and accompanying wash steps was that the fluidMAG-Amine-glutaraldehyde-protein sample was easier to separate through magnetic decantation and stayed in solution for longer periods before insolubilization than the accompanying EDC and protein adsorption samples. This could suggest that the fluidMAG-Amine-glutaraldehyde-E537D β-Gal is more stable than the fluidMAG-Amine-EDC-E537D β-Gal.

The protein determination data of the separate detergent wash steps were used together with the previous protein determination data of section 5.3.1 to determine the saturation of 1 mg FluidMAG-Amine with E537D β-Gal via EDC coupling or glutaraldehyde cross-linking (Figure 5.4 and 5.5).

The maximum amount of E537D β-Gal that could be immobilised to 1 mg fluidMAG-Amine via EDC coupling was 55 μg (Figure 5.4) while saturation of 1 mg fluidMAG-Amine via glutaraldehyde cross-linking was less clear but reached at 80 μg (Figure 5.5). This is consistent with the the higher concentration of protein that remained in the supernatant after immobilisation via EDC coupling as previously mentioned. It is therefore evident that more E537D β-Gal could be covalently immobilised to fluidMAG-Amine via glutaraldehyde cross-linking, compared to EDC coupling. This phenomenon may be due to the multi-component functional nature of gluteraldehyde that proposes a variety of reactive forms that allows for much for β-Gal to be linked to the fluidMAG-Amine compared to EDC, which can only accommodate one enzyme molecule per reactive intermediate. Significantly more physical adsorption also occurred with the EDC coupling strategy as compared to the

glutaraldehyde cross-linking strategy, Figure 5.4 and 5.5.

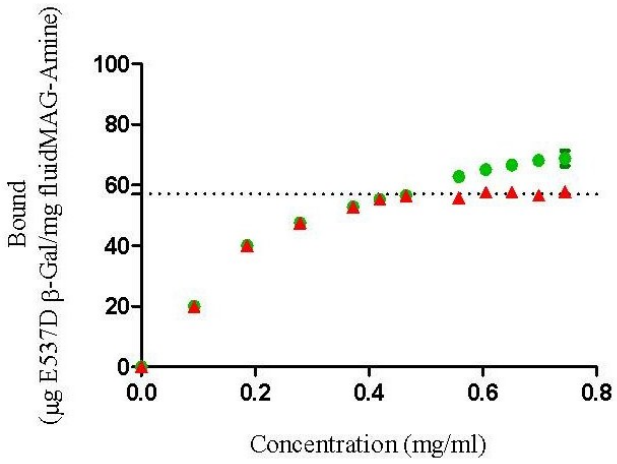


Figure 5.4: Saturation of 1 mg fluidMAG-Amine via EDC coupling before and after the desorption of adsorbed E537D β-Gal as calculated by GraphPad Prism 5 software. Concentration on x-axis refers to the protein concentration in Table 5.1. ● : Before desorption of adsorbed protein, △ : After desorption of adsorbed protein. Error bars indicate SEM, n = 3.

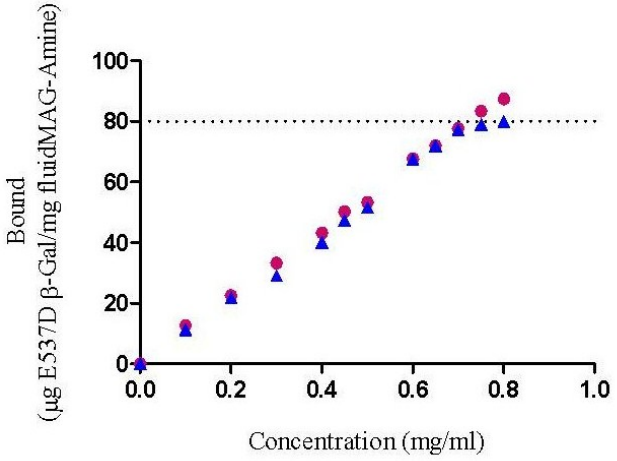


Figure 5.5: Saturation of 1 mg fluidMAG-Amine via glutaraldehyde cross-linking before and after the desorption of adsorbed E537D β-Gal as calculated by GraphPad Prism 5 software. Concentration on x-axis refers to the protein concentration in Table 5.2. ● : Before desorption of adsorbed protein, △ : After desorption of adsorbed protein. Error bars indicate SEM, n = 3.

5.3.3 ATR-FTIR spectra

ATR-FTIR analysis was performed on the protein coated MNPs before and after the desorption of adsorbed protein and the absorbencies of the different samples collected and compared using OMNIC software (Figure 5.6). Figure 5.6 shows the amide peak absorbances in the infrared for these samples. The amide peak absorbance range is 1680 - 1630 cm^{-1} for the C=O stretch whereas N-H bending occurs in the region of 1640 - 1550 cm^{-1} for primary (I) and secondary (II) amides, as labelled in Figure 5.6. The N-H stretch in primary amides (-NH_2) gives two bands near 3350 and 3180 cm^{-1} whereas secondary amides have one band (-NH) at about 3300 cm^{-1}.

Almost all the protein adsorbed to the fluidMAG-Amine surface for the control sample with untreated fluidMAG-Amine (Figure 5.6(a) turquoise spectrum) was removed during the subsequent SDS detergent wash step (Figure 5.6(a) red spectrum) when compared to the fluidMAG-Amine control (Figure 5.6(b)). From this observation, where all the non-covalently coupled protein was successfully removed during the detergent wash for the control sample, it was assumed that the detergent wash step would remove any possible non-covalently coupled protein from all the samples.

The presence of amide peaks in the EDC and glutaraldehyde spectra, after the detergent wash, is indicative of covalent linkages. From this results it was, therefore evident that E537D β-Gal was covalently attached to fluidMAG-Amine. It was, however, evident that some physi-sorption did occur with both the EDC and glutaraldehyde methods as was evident when comparing the before and after desorption graphs in section 5.3.2 and when comparing the before and after desorption FTIR spectra in Figure 5.6.

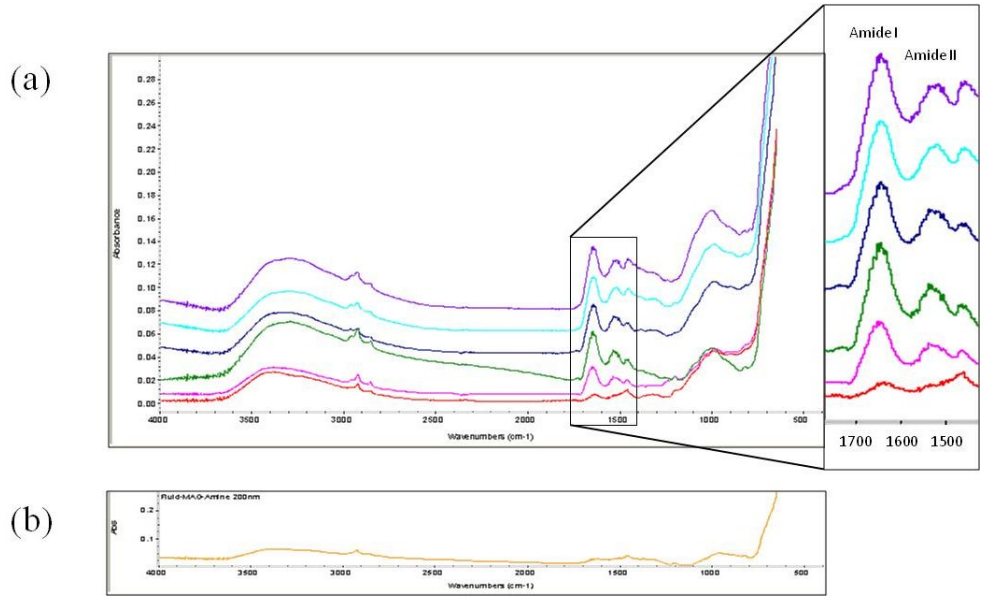


Figure 5.6: Infrared (ATR-FTIR) spectra of E537D β-Gal immobilised on fluidMAG-Amine through EDC coupling, glutaraldehyde cross-linking or physical adsorption. (a) Protein attached during incubation on the glutaraldehyde treated fluidMAG-Amine (purple spectrum, unshifted at top) is retained after SDS washing (dark green spectrum, unshifted in fourth position) [2 % SDS solution, 50 ^{o}C for 1 hour]. Protein attached during incubation with EDC and fluidMAG-Amine (dark blue spectrum, unshifted at third position) is also retained after SDS washing (pink spectrum, unshifted in fifth position). Protein attached during incubation on the untreated fluidMAG-Amine (turquoise spectrum, unshifted at second position) is almost completely removed after SDS washing (red spectrum, unshifted at bottom) when compared to (b) pure 200 nm fluidMAG-Amine.

5.3.4 TEM images

Measurements of the uncoated control fluidMAG-Amine particles made during TEM analysis indicated these particles to be roughly between 8 and 15 nm in diameter (Figure 5.8). The results from this TEM analysis of the fluidMAG-Amine is very similar to a TEM image of the fluidMAG-Amine (200 nm) that was requested and received from Chemicell (Berlin, Germany), Figure 5.7.

The protein coating of the particles via EDC and glutaraldehyde is not that clearly visible from the TEM images shown below (Figures 5.9 and 5.10). However, it appears as though these coated particles tend to be more clustered (darker due to more layers) compared to the uncoated particles and it is plausible that this phenomenon is due to protein-protein and protein-particle cross-

linking that occurred during the coupling step. It is also impossible to detect any significant difference between the two immobilization strategies from these TEM images.

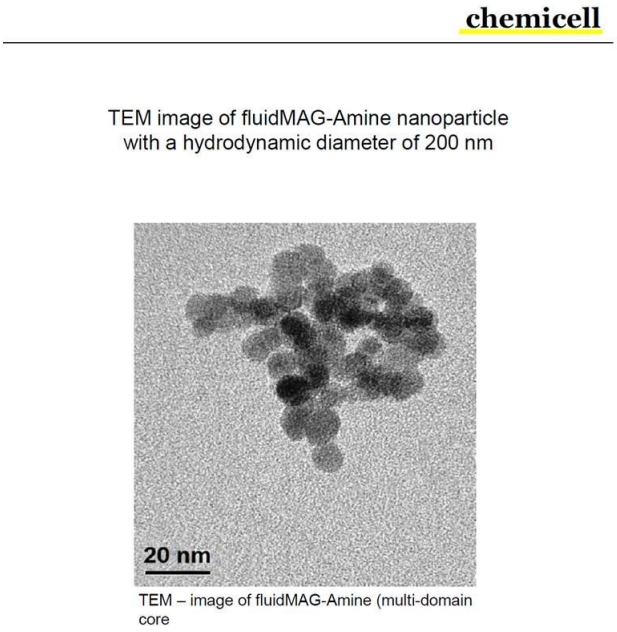


Figure 5.7: TEM image requested from Chemicell (Berlin, Germany).

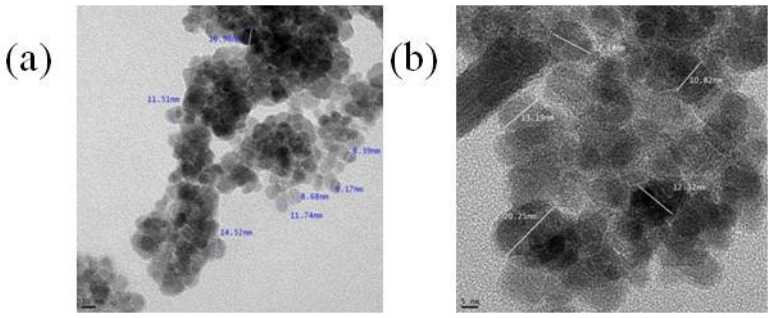


Figure 5.8: TEM images of uncoated fluidMAG-Amine particles (a) 200 000 X magnification (b) 470 000 X magnification with the size of the uncoated particles calculated to be approximately 10 nm in diameter.

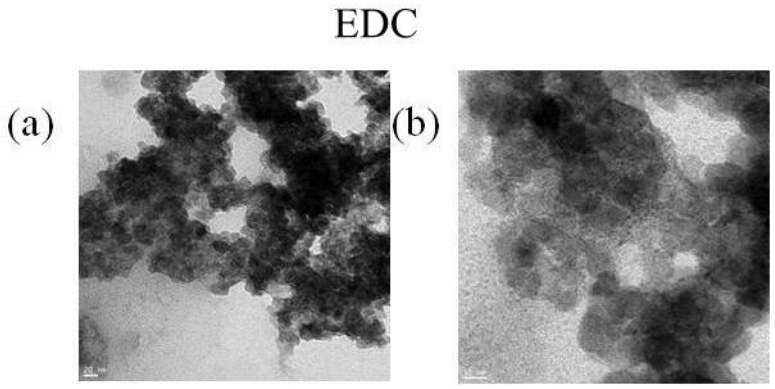


Figure 5.9: TEM images of E537D β-Gal coated fluidMAG-Amine via EDC coupling (a) 100 000 X magnification (b) 340 000 X magnification.

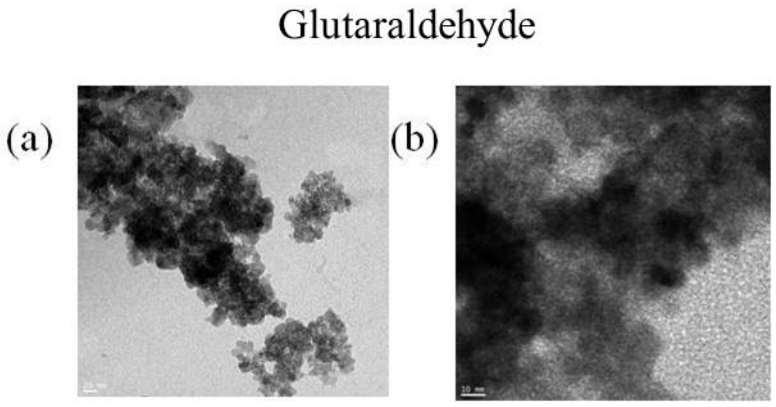


Figure 5.10: TEM images of E537D β-Gal coated fluidMAG-Amine via glutaraldehyde cross-linking (a) 100 000 X magnification (b) 340 000 X magnification.

5.3.5 SEM images

SEM images of the uncoated and coated fluidMAG-Amine particles revealed spherical particles with a size range between 50 and 80 nm. Unfortunately, due to the wide variation in particle size, an increase in size due to protein coating of the particles could not be calculated. Interestingly, the measurements of the uncoated particles from the TEM analysis (Figure 5.8, 8 - 15 nm) and the measurements taken during SEM analysis (Figure 5.11, 50 - 80 nm) were very different. Both these analysis were performed on the same original batch of fluidMAG-Amine. It is, however, possible that during TEM analysis, the electron beam only registered the core magnetite material of the particles and

not the aminosilane matrix, while the electron beam during SEM analysis scanned the surface, including the aminosilane matrix, during the analysis. Another possible explanation could pertain to calibration differences between these two instruments.

In the images of the coated particles (Figures 5.12 and 5.13), a thin layer coating the surface of the particles can be observed when compared to the uncoated particles (Figure 5.11). Single particles are then also less distinguishable and the particles appear even more clustered than the uncoated ones. The latter may be primarily due to the protein-particle cross-linking that was intended during the coupling step, as well as possible protein-protein cross-linking that was not intended as such, but most probably occurred. Again, no significant difference between EDC and glutaraldehyde can be seen.

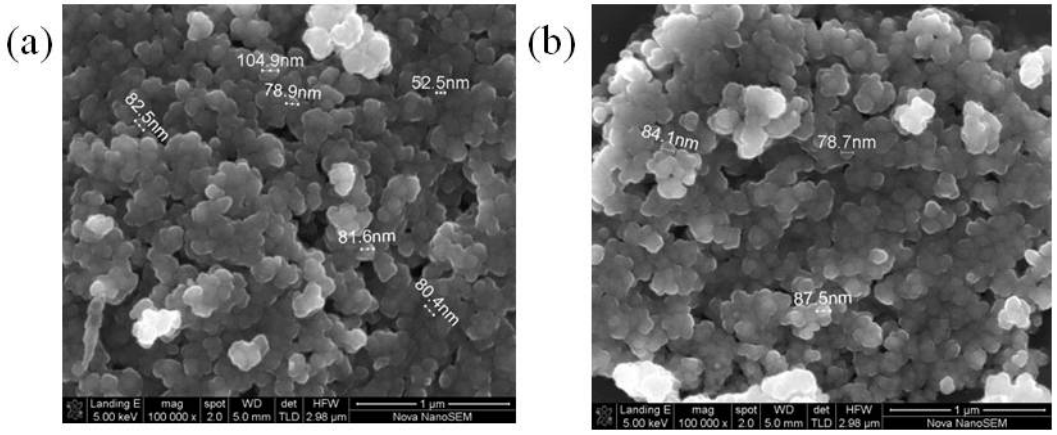


Figure 5.11: SEM images of uncoated fluidMAG-Amine particles of two areas (a and b) magnified by 100 000 with the size of the uncoated particles calculated to be 50 - 80 nm in diameter.

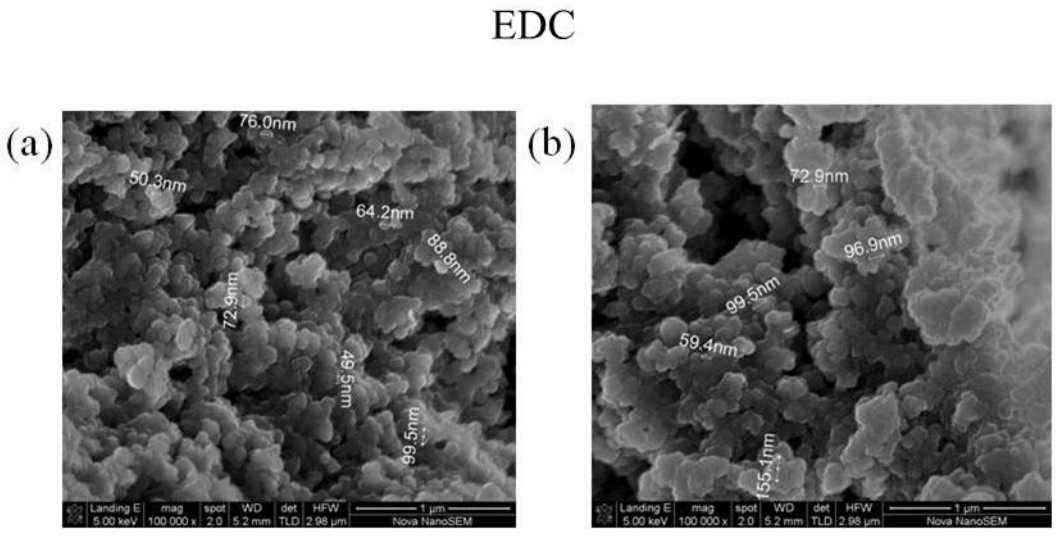


Figure 5.12: SEM images of E537D β-Gal coated fluidMAG-Amine particles via EDC coupling of two areas (a and b) magnified by 100 000.

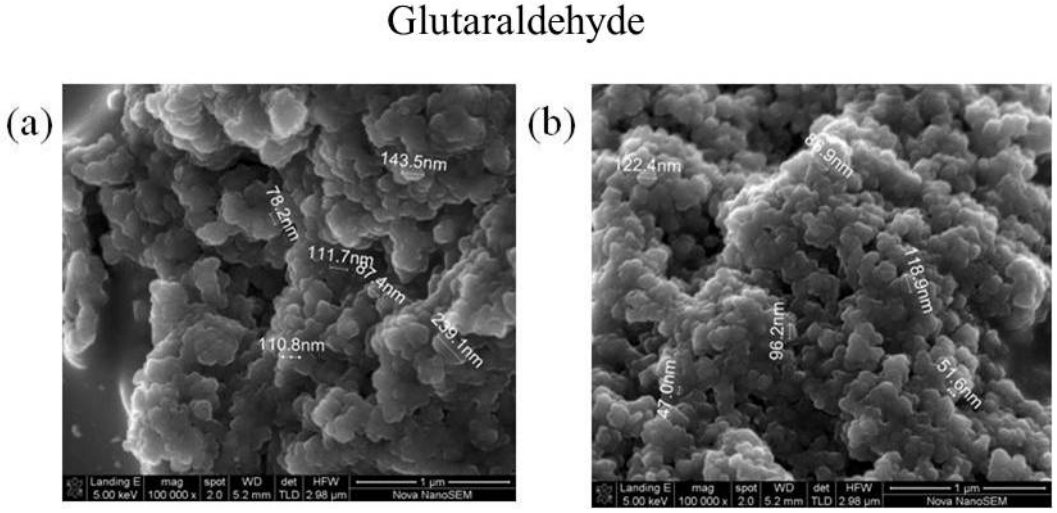


Figure 5.13: SEM images of E537D β-Gal coated fluidMAG-Amine particles via glutaraldehyde cross-linking of two areas (a and b) magnified by 100 000.

5.4 Conclusion

The random covalent immobilisation of E537D β-Gal to fluidMAG-Amine via EDC coupling and glutaraldehyde cross-linking was investigated and confirmed by a series of analyses.

BCA protein determination assays indicated a loss in protein present in the supernatant, used for the coupling step, after immobilisation compared to

before immobilisation. A detergent wash step, with a strong ionic surfactant, SDS, indicated covalent protein attachment to the MNPs. This was evident from the detergent's ability to only remove or separate a small amount of the protein from the MNPs. The amount of protein desorbed by the detergent only increased significantly when 55 μg E537D β-Gal was reached for the EDC coupling method and 80 μg for the glutaraldehyde cross-linking strategy (Figures 5.4 and 5.5). ATR-FTIR analysis on these protein coated particles further confirmed the presence of a covalent amide bond between the protein and the MNPs (Figure 5.6). Although TEM and SEM analysis did not provide clear images regarding protein surface coating of the MNPs, it was possible to detect a difference between the uncoated and protein coated particles. This is therefore sufficient evidence to confirm the covalent immobilisation of E537D β-Gal to fluidMAG-Amine via EDC coupling and glutaraldehyde cross-linking. Furthermore, it was calculated that the glutaraldehyde cross-linking strategy enabled an increased carrier capacity of E537D β-Gal protein to fluidMAG-Amine compared to the EDC coupling strategy.

It is noteworthy to mention here that these cross-linking strategies (EDC and glutaraldehyde) result in strong, covalent bonds that cannot be reversed under normal conditions and would prevent enzyme leakage from the MNPs.

The final factor that was, however, to be determined, was whether the EDC and glutaraldehyde immobilised E537D β-Gal to fluidMAG-Amine had retained their substrate binding ability and enzymatic activity. This aspect would be investigated through immobilised binding assays in Chapter 6.

Chapter 6

THE CHARACTERIZATION AND OPTIMIZATION OF COVALENTLY IMMOBILISED E537D β-GAL ACTIVITY

6.1 Introduction

The successful covalent immobilisation of E537D β-Gal to fluidMAG-Amine, via two coupling strategies i.e. EDC coupling and glutaraldehyde cross-linking, was discussed in Chapter 5. The covalent immobilisation of enzymes usually results in some loss of activity due to the strong permanent bond that may influence enzyme structure, substrate binding ability and catalytic function. The next step was, therefore, the characterization of the immobilised enzyme-particle complexes with regards to retained enzymatic activity in order to determine the ultimate binding potential for the removal of lactose from solution.

As described previously, a detergent wash step was employed to desorb any non covalently bound enzyme from the fluidMAG-Amine. For this purpose

SDS was used as described by Van den Berg *et al.* [95]. SDS is, however, a strong ionic surfactant that unfolds and denatures protein structure, usually resulting in a loss of enzymatic activity. It was therefore necessary to determine how SDS would influence the enzymatic activity of β-Gal as well as to compare SDS to alternative detergents for the same purpose.

Enzymatic characterization, of the covalently immobilised enzyme, included the testing of the ligand binding ability with two β-Gal ligands, ONPG and lactose, and thereafter the hydrolysis of the substrate (retained enzymatic activity) to the accompanying products. The E537D mutated version of the enzyme has a much lower enzymatic activity compared to the wildtype β-Gal, as described in section 3.3.4. This would, therefore, allow the binding and removal of lactose from milk accompanied by the hydrolysis of part of the lactose to glucose and galactose (low enzymatic activity) to compensate for the loss in sugar.

An immobilised form of the expressed and purified wildtype β-Gal enzyme was included during the activity optimization and characterization experiments as a control.

6.1.1 Carbohydrate analysis

Carbohydrates occur in many food products and some carbohydrates can also be used as food additives. A whole range of detection and analysis methods for carbohydrates have been developed specifically for monitoring the carbohydrate content of foodstuffs. These, specifically developed for the qualitative and quantitative analysis of carbohydrates, can be divided into chromatographic [105], electrophoretic [106; 107], chemical (colorimetric, titration and gravimetric), enzymatic activity (catalysis of specific reactions) and physical (infrared, density, refractive index and polarimetry) methods as well as immunoassays [108]. The chromatographic methods are the most frequently used to determine the type and concentration of mono- and oligosaccharides in

foodstuffs. Chromatography allows for rapid, repeatable separation and quantification of a wide range of carbohydrates. Chromatography, together with radiolabeled substrates, was therefore used to characterize β-Gal activity in this study. A short overview of these technologies will subsequently be given as background.

6.1.1.1 Chromatography

Chromatographic methods used for carbohydrate analyses include gas chromatography (GC), thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The final separation and identification of carbohydrates is achieved by means of their partition coefficients, sizes or polarities depending on the stationary phase employed.

Gas Chromatography

Analysis by GC is based on the separation of a mixture of components according to the degree of affinity for or interaction of each component with a liquid stationary phase. During GC analysis the components are vaporized in a gas phase and separations occur at high temperatures. Components in the mixture to be separated that express a higher affinity for the stationary phase would elute later while those with less of an affinity for the stationary phase would elute first.

The GC analysis of carbohydrates is an established technique and one of the main advantages is the small sample size that is required. Sample preparation for GC analysis of carbohydrates is, however, laborious and involves several steps, including derivitization [109], and may result in sample loss.

According to Rogatsky and Stein [110], routine quantitative GC accompanied by mass spectrometry (MS) analysis of carbohydrates are usually not preferred because it requires multi-step sample preparation, pre-purified analytes and long runtimes.

Thin layer Chromatography

For TLC, the separation of analytes occur on a stationary phase that usually consists of a thin layer coating on a square or rectangular, metal foil or glass plate [105]. The separation can be based on either partition, adsorption, ion-exchange, chiral or molecular exclusion principles depending on the stationary phase selected. The test sample is placed as either a spot or a thin band near one end of the plate, whereafter this end of the plate is then placed in a reservoir containing the mobile phase [105]. Naturally, due to capillary action, the mobile phase passes over the plate until it is removed from the reservoir or until the mobile phase front reaches the end of the plate [105]. Analytes in the test sample are ultimately separated at a rate determined by their distribution coefficients between the mobile and stationary phases. Here, the movement of a given analyte is characterized by a retardation factor (R_f) [105]. The main advantage of TLC is the speed and flexibility whereby a number of constituents can be determined while, on the other hand, results are difficult to reproduce.

High performance liquid Chromatography

HPLC analysis is where components of an applied sample are separated by the continuous passage of the liquid mobile phase through the column containing a stationary phase consisting of small particles. The small particles are developed to withstand high pressures that result from back-pressure in the column due to the great resistance to flow of the mobile phase [105]. The columns generally used in HPLC analyses are made from stainless steel and manufactured to withstand pressures of up to 5.5 X 10^7 Pa [105]. These are then also further classified as either conventional or microbore columns where microbore columns have several advantageous including reduced eluent consumption due to slower flow rates and increased sensitivity [105]. The small particle matrices/stationary phase material, specifically developed to withstand high pressures, are mostly spherical particles of uniform size and classi-

fied as either microporous supports, pellicular (superficially porous) supports or bonded phases [105].

The mobile phase to be used to achieve sufficient separation of analytes during HPLC analysis can be either isocratic elution or gradient elution depending on the separation required. It is also essential that all eluents be degassed before use to prevent air bubbles in pumps [105].

HPLC has become the preferred method for routine carbohydrate analyses due to its separation and quantification abilities, accuracy and rapidity but is limited due to the absence of a chromophore in carbohydrates [111]. For carbohydrate analysis, the detection methods usually coupled to HPLC are refractive index (RI) detection, MS detection and pulsed amperometric detection (PAD) [112]. For RI the concentration detection limit is around 10^{-1} ppm and for PAD it is more or less 10^{-2} ppm [112].

6.1.1.2 Radiolabeling and isotopic markers

Radiolabeled carbohydrates are commonly used to investigate the quantity and properties of carbohydrates, especially after chromatographic separation. ^{14}C labeled mono-, di-, or oligosaccharides are commercially available and radiation studies with these provide a sensitive and relatively straight forward alternative to investigate the *in vitro* metabolism [113], fermentation [114] and general properties of carbohydrates. In this study, radiolabelled lactose will be used to investigate the substrate binding ability and remaining activity of the immobilised enzyme.

6.2 Materials and Methods

6.2.1 Reagents and chemicals

Wildtype and E537D β-Gal were covalently immobilised to fluidMAG-Amine (200 nm) through glutaraldehyde and EDC strategies, as previously described. Equal amounts of β-Gal (55 μg, 0.090 ml of 1 mg/ml), whether the wildtype or the mutated version, were immobilised to 1 mg fluidMAG-Amine using both glutaraldehyde cross-linking and EDC coupling prior to activity investigations.

Radiolabeled Lactose (D-glucose-1-^{14}C) (A*merica Radiolabeled Chemicals, Inc.*, St.Louis) and a Sugar-Pak I P/N 85188 column (Millipore, WATERS (WAT085188)) as well as ONPG (*Sigma-Aldrich Chemical Co.*, Germany) were purchased for characterization purposes. All chemicals used for the preparation of buffers and solutions were of analytical grade or higher and purchased from *Merck*, *Sigma-Aldrich Chemical Co.* or *Bio-Rad*. An Ultra Pure Milli-Q water system was used to produce reagent grade water used in all buffers and experiments

6.2.2 Detergent wash optimization

As mentioned previously, SDS is a strong detergent and might result in protein denaturation and loss of enzymatic activity. Two other less stringent detergents, Tween-20 and Tween-80, were therefore compared to SDS for the desorption of non specific adsorbed protein.

For this purpose, the purified wildtype β-Gal was used because the enzymatic activity is much higher when compared to the mutated enzyme and as a result the colour reaction, with ONPG as substrate, is more easily visible to the naked eye.

Three groups of immobilised samples, each consisting of six glutaraldehyde cross-linked wildtype β-Gal to fluidMAG-Amine samples as well as six EDC coupled wildtype β-Gal to fluidMAG-Amine samples, were prepared as

described in Chapter 5, 55 μg of protein immobilised to 1 mg fluidMAG-Amine. After the completion of the coupling and accompanying wash steps, the samples were washed with the detergents in duplicate, therefore, two glutaraldehyde immobilised samples of each group were washed with SDS, two with Tween-20 and two with Tween-80, using the same protocol. The identical protocol was followed for the three groups of six EDC immobilised samples. The detergent wash was therefore performed with a 2 % solution of either SDS, Tween-20 or Tween-80 in PBS buffer, pH 7.4, whereas the incubation times and temperatures of the three groups of samples differed. It was 1 hour with gentle mixing at either ambient temperature or 50 ^{o}C or overnight at 4 ^{o}C.

Afterwards, the protein-fluidMAG-Amine complexes were separated from the detergent wash solution through magnetic decantation and the wash solution collected for a BCA protein determination assay. The protein-fluidMAG-Amine complexes were washed with, and resuspended, in PBS buffer, pH 7.4 (100 μl), before the activity assays were conducted.

For the activity assays, ONPG, at a final concentration of 13.3 mM, dissolved in Novo buffer pH 6.5 (Novo, commercial information 1979) was used. NOVO buffer is a buffer designed to reproduce the composition of milk (2.7 mM sodium citrate, 7.91 mM citric acid, 2.99 mM potassium biphosphate, 10.84 mM potassium phosphate, 19.43 mM potassium hydroxide, 4.08 mM magnesium chloride, 5.1 mM calcium chloride and 3.33 mM sodium carbonate) [59]. β-Gal enzymatic activity results in the same colour reaction previously mentioned in section 3.2.4. The colour change is visible with the naked eye and can be measured as absorbance at 420 nm.

The suspended protein-fluidMAG-Amine complexes (undiluted[1]) were incubated with the NOVO buffer containing ONPG (120 μl) for 10 minutes at

[1]The reason for using undiluted protein-fluidMAG-Amine complexes was the following: it is impossible to dilute the complexes, have a uniform dispersion and repeatably pipette equal amounts of the diluted complexes due to the continuous precipitation of the particles. The assay absorbencies for the different detergent washes was, however, comparable since the assay conditions were kept constant for all the samples.

37 °C whereafter the complexes were separated from the supernatant through magnetic decantation and the supernatant added to a microplate and analyzed at 420 nm. For the assay, a blank, as well as a unimmobilised wildtype β-Gal incubated with the substrate solution under the same conditions, were included as a reference for enzymatic activity.

It is important to note that the unimmobilised/free enzyme could not be separated from the assay buffer and was therefore still present and active in the microplate well. The suspended protein-fluidMAG-Amine complexes were not diluted as previously performed for the wildtype β-Gal ONPG assay (refer to section 3.2.4).

6.2.3 Immobilised wildtype and E537D β-Gal radioactive binding assay with radiolabelled lactose (D-glucose-1-^{14}C)

The immobilised radioactive binding assay was conducted similar to the assay previously described by Dodd [4]. FluidMAG-Amine EDC and glutaraldehyde immobilised wildtype and E537D β-Gal samples were incubated with a 0.2 nmol radiolabeled lactose (D-glucose-1-^{14}C) solution in NOVO buffer, pH 6.5, for 5 minutes at room temperature in triplicate. The immobilised enzyme-particle samples were then removed from the solution via magnetic decantation. The original 0.2 nmol radiolabeled lactose solution as well as the residual supernatant solution, after incubation with the immobilised enzyme-particles, were analyzed with a scintillation counter. Several experimental control samples were also included i.e. samples containing fluidMAG-Amine but no protein, samples not incubated with D-glucose-1-^{14}C as well as a wash step of all the samples after incubation with D-glucose-1-^{14}C to remove any non-specific binding of D-glucose-1-^{14}C to the enzyme.

6.2.4 Immobilised enzymatic assay with ONPG

The presence of activity in the EDC and glutaraldehyde immobilised wildtype β-Gal was indicated with ONPG, at a final concentration of 13.3 mM, dissolved in Novo buffer, pH 6.5, at 37 ^{o}C for 5 minutes.

As previously mentioned, the unimmobilised/free enzyme (wildtype β-Gal) could not be separated from the assay buffer and remained active in the microplate well. This was therefore not an accurate comparison between free and immobilised enzyme for the calculation of retained enzymatic activity but the two immobilisation strategies could be compared due to consistent experimental conditions.

6.2.5 Radioactive Partition HPLC

The lactose hydrolysis activity of the immobilised wildtype and E537D β-Gal, as well as the mutant's potential to physically remove lactose from a lactose containing solution, was determined by Radioactive Partition HPLC analysis. All HPLC analyses were performed on a SpectraSYSTEM P4000 high performance liquid chromatograph (Thermo Separation^{TM} products, San Jose, CA, USA) coupled to a SpectraSYSTEM AS3000 automatic injector (Thermo Separation^{TM} products, San Jose, CA, USA) and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL, USA). A Sugar-Pak I column was used and operated at 70 ^{o}C. The HPLC was controlled with ChromQuest version 2.53 ((1998-1999) ThermoQuest Corporation) computer software.

The column was equilibrated according to manufacturer's instructions with a 0.001 M calcium EDTA solution (500 mg/L)(100 ml) at a flow rate of 0.5 ml/min at 70 ^{o}C. The mobile phase for sample analysis was a solution of 0.1 mM calcium EDTA (50 mg/L) kept between 70 and 80 ^{o}C and operated at a flow rate of 0.6 ml/min. The scintillation fluid was added at a flow rate of three times that of the mobile phase for radioactive detection.

The activity assays were carried out as described in section 6.2.3 with the residual supernatant analyzed by HPLC instead of the scintillation counter.

6.3 Results and Discussion

6.3.1 Investigation into the most efficient detergent for the desorption of non-specific adsorbed protein

Activity of the suspended protein-fluidMAG-Amine complexes were investigated with ONPG as substrate, dissolved in NOVO buffer, pH 6.5, at 37 ^{o}C for 10 minutes and the activity measured as absorbance at 420 nm. Figure 6.1 is an evaluation of the three detergents used for the desorption of adsorbed protein and compared regarding the percentage retained enzymatic activity per mg protein immobilised in contrast to the enzymatic activity of the unimmobilised enzyme.

Enzymatic activity of all the detergent washed samples did not appear to change significantly when the wash step was performed at ambient temperature, however, when the wash step was performed at either 4 ^{o}C or 50 ^{o}C, the activity was influenced significantly. At 50 ^{o}C the 2 % SDS wash denatured the enzyme resulting in a complete loss of enzymatic activity (Figure 6.1). Reaction conditions of 4 ^{o}C overnight influenced not only the SDS washed sample but also the activity of the EDC coupled Tween-washed samples. Interestingly the glutaraldehyde cross-linked samples retained their activity.

These results revealed Tween-20 and -80 to be less detrimental than SDS regarding retained enzymatic activity after wash steps performed at 4 ^{o}C, ambient temperature and 50 ^{o}C. According to Van den Berg *et al.* [95], SDS has the "best detergency power" and is able to remove most or all of the adsorbed protein; Tween-80 on the other hand has very good detergent properties for the prevention of protein adsorption, especially when included with glutaraldehyde

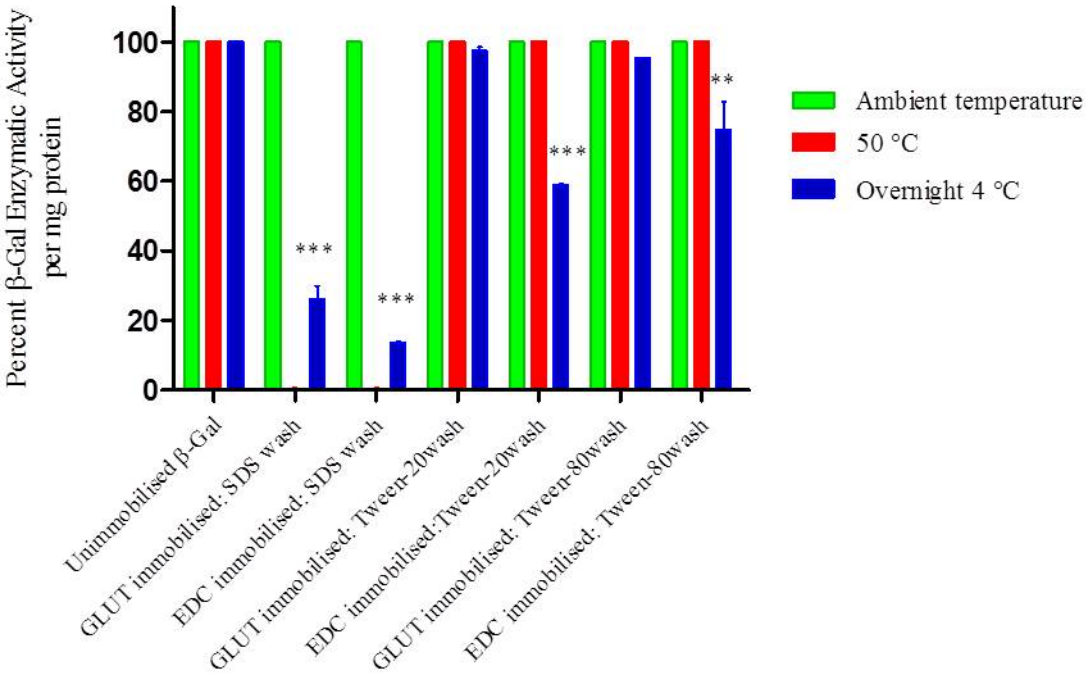


Figure 6.1: Comparison between SDS, Tween-20 and Tween-80 regarding the percent retained activity for the desorption of adsorbed protein after incubations at 4 ^{o}C, ambient temperature and 50 ^{o}C. Graphpad Prism 5 software was used for statistical analysis. Columns were compared to the control column by one-way ANOVA, followed by Dunnett's post test (***P $<$ 0.05). GLUT = glutaraldehyde. Error bars indicate SEM, n = 2.

in the reaction mixture. For the subsequent assays, Tween-20 was used as detergent to desorb physically adsorbed protein.

6.3.2 Radioactive binding assay with radiolabelled lactose (D-glucose-1-^{14}C)

Radioactive binding assay data received from the scintillation counter were compared regarding the percentage lactose bound per immobilised protein sample and are summarized in Figure 6.2. Several controls were included, as mentioned previously in section 6.2.3, to ensure reproducibility.

From the results summarized in Figure 6.2 it is evident that there is a

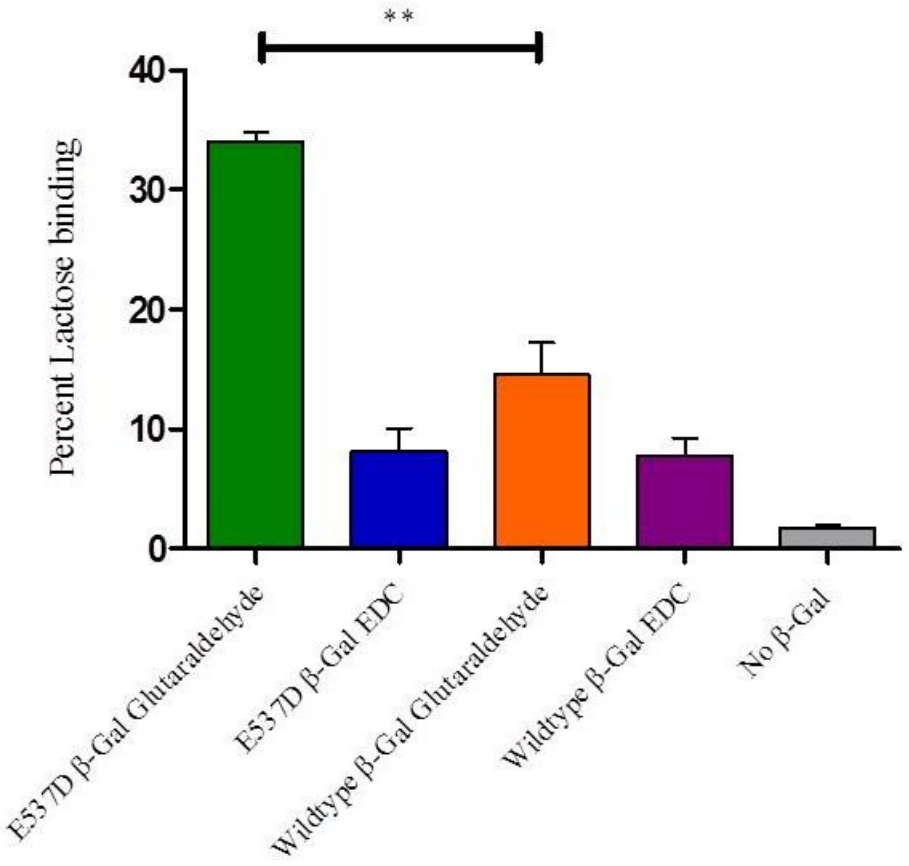


Figure 6.2: Comparison between percent lactose binding per immobilised protein sample via the two immobilisation strategies. The E537D β-Gal immobilised via glutaraldehyde was able to bind approximately 34 % of the lactose present while the E537D β-Gal immobilised via EDC was only able to bind approximately 8 % of the lactose present. The wildtype β-Gal immobilised via glutaraldehyde and EDC was also able to bind some of the lactose present. Graphpad Prism 5 software was used for statistical analysis. Columns for the glutaraldehyde immobilised E537D and wildtype β-Gal were compared by one-way ANOVA, followed by a t test (**P < 0.05). Error bars indicate SEM, n = 3.

significant difference between the amount of lactose that E537D β-Gal, immobilised via glutaraldehyde, can bind for prolonged periods (5 - 10 minutes) as compared to that of the immobilised wildtype that has a significantly higher catalytic activity, which means the substrate is converted to the products as soon as it is located to the active site. In addition, the glutaraldehyde immobilised E537D β-Gal could bind approximately 34 % of 0.2 nmol lactose while the EDC immobilised E537D β-Gal could only bind approximately 8 % of the lactose present. This suggests that the glutaraldehyde strategy for

the covalent immobilisation of E537D β-Gal may result in a more functional immobilised enzyme, capable of binding lactose more efficiently than the EDC immobilised strategy, when equal amounts of enzyme were immobilised to fluidMAG-Amine. This may also explain the difference that can be seen, in Figure 6.2, with glutaraldehyde between the mutant and wildtype, which is not present in the EDC samples. Here, the EDC immobilised E537D β-Gal has lost some of its substrate binding capabilities, possibly due to conformational changes induced by the "zero-length" covalent coupling to the MNPs.

6.3.3 Immobilised enzymatic activity assay with ONPG

Observations during the assay indicated that the glutaraldehyde cross-linked enzyme-particles retained more enzymatic activity when compared to the EDC coupled enzyme-particles (yellow colour visible with the naked eye). This observation was confirmed by a spectrophotometric measurement at 420 nm.

Figure 6.3 is a representation of the percentage retained activity, which is comparable between the two immobilisation strategies. The data indicated that the glutaraldehyde cross-linked enzyme-particles retained more than twice the percentage retained enzymatic activity of the EDC coupled enzyme-particles. This again suggested that the glutaraldehyde immobilisation strategy might result in a more functionally immobilised enzyme compared to the EDC immobilisation strategy for the coupling of β-Gal to fluidMAG-Amine.

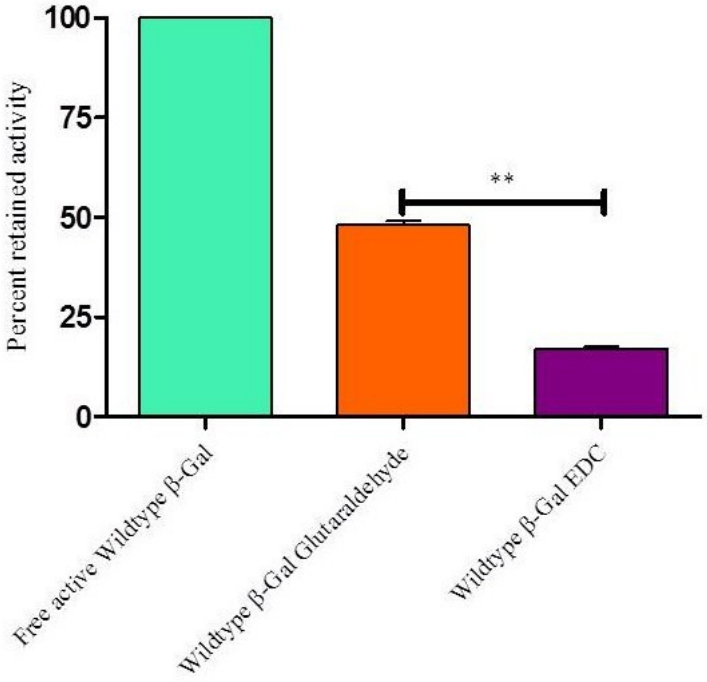


Figure 6.3: Comparison between percentage retained activity between glutaraldehyde and EDC immobilised wildtype β-Gal as investigated with ONPG as substrate at 420 nm. Graphpad Prism 5 software was used for statistical analysis. Columns were compared by a t test (**P $<$ 0.05). Error bars indicate SEM, n = 3.

6.3.4 Immobilised enzymatic activity assay with radiolabelled lactose (D-glucose-1-^{14}C) - Radioactive Partition HPLC

Partition HPLC analysis with radioactive detection was used to separate the radiolabeled lactose (disaccharide sugar) and glucose (monosaccharide sugar) to allow distinction between the removal of lactose by the enzyme-particle complexes and hydrolysis to glucose and galactose.

6.3.4.1 Determination of retention times

The Sugar-Pak I column used for the separation of lactose and glucose in this project, is a column generally used for the analysis of sugar products. The column can be used to separate many monosaccharides, polyols and alcohols [115]. Larger sugars and disaccharides can also be separated according

to molecular weight [115]. The 300 x 6.5 mm column was packed with a microparticulate cation-exchange gel in calcium form and the manufacturer instructions regarding proper handling, storage and operation were closely followed. The chromatography of the ^{14}C glucose standard, after separation on the Sugar-Pak column, resulted in two peaks, one at 4.6 min and one at 8.3 min, while the retention time of ^{14}C lactose (D-glucose-1-^{14}C) was 6.67 min as shown in Figure 6.4. The two peaks observed after ^{14}C glucose separation can be ascribed to the two naturally occurring α-D- and β-D-isomers present at high temperatures.

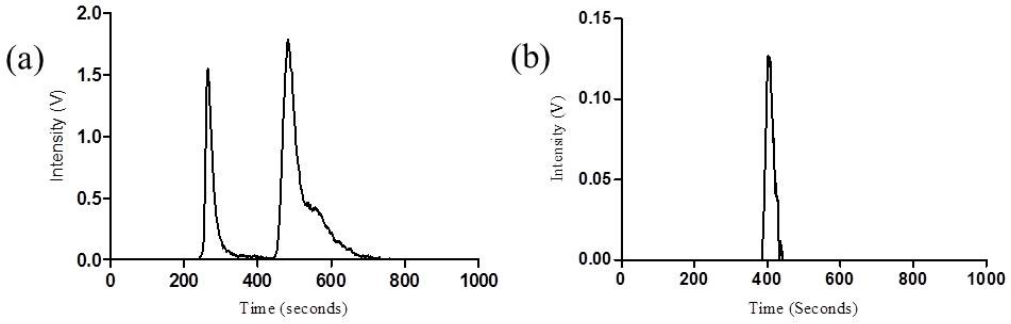


Figure 6.4: Partition HPLC chromatograms of (a) ^{14}C glucose and (b) ^{14}C lactose on a Sugar-Pak I column operated between 70 and 80 ^{o}C with 0.1 mM calcium EDTA as mobile phase.

6.3.4.2 Partition HPLC of radioactive binding assay control and supernatant samples

Supernatant samples of the radioactive binding assay, as explained in section 6.2.3, were added to HPLC autosampler viles (12 x 32 mm) fitted with inserts and 8 mm septa. The partition of each sample was compared to the partition of an experimental control i.e. the supernatant after incubation of 0.2 nmol radiolabeled lactose (D-glucose-1-^{14}C) with uncoated "naked" fluidMAG-Amine. Consequently, any difference observed in carbohydrate separation or detected radioactive counts between the supernatant samples and the control can be

ascribed to the particular β-Gal coating, whether it is the E537D or wildtype β-Gal.

The calculated integrated peak area of lactose, obtained with partition HPLC separation of the experimental control sample, shown in Figure 6.5, averaged at 31486 CPM as calculated by the HPLC Flow-One/Beta Data Acquisition version 2.0 ((19/08/2008) K. Visser) computer software program. This was taken as 100 % for the subsequent calculations of the percentage lactose bound.

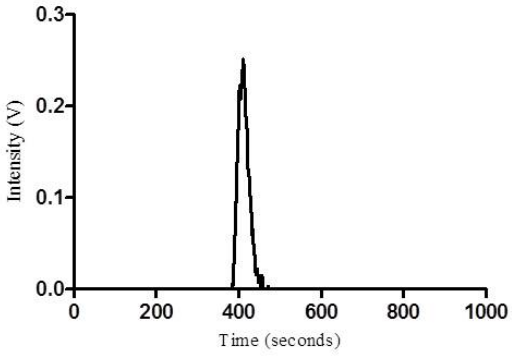


Figure 6.5: Partition HPLC chromatogram of the radiolabeled lactose experimental control sample on a Sugar-Pak I column operated between 70 and 80 °C with 0.1 mM calcium EDTA as mobile phase.

The chromatograms of the partition HPLC separation of the supernatant samples obtained after the incubation of radiolabeled lactose with the fluidMAG-Amine EDC and glutaraldehyde immobilised wildtype and E537D β-Gal samples are shown in Figure 6.6(a)-(d). The supernatant removed from the fluidMAG-Amine containing the immobilised E537D β-Gal (Figure 6.6(a) EDC and (b) Glutaraldehyde) yielded chromatograms with single peaks that agree with the retention time of lactose. The absence of additional peaks in the chromatograms indicate that no detectable hydrolysis of lactose occurred during this 5 minute incubation. In contrast, the supernatant removed from the fluidMAG-Amine containing the immobilised wildtype β-Gal (Figure 6.6(c) EDC and

(d) Glutaraldehyde) yielded chromatograms with peaks that agree with the retention times of only the glucose isomers. This suggests that the 5 minute incubation period with the immobilised wildtype samples allowed complete hydrolysis of the 0.2 nmol lactose that was present in the solution.

The calculated integrated peak area for the EDC immobilised E537D β-Gal averaged at 28646 CPM compared to 21253 CPM for the glutaraldehyde immobilised E537D β-Gal. These values were used to calculate the percent lactose binding per sample.

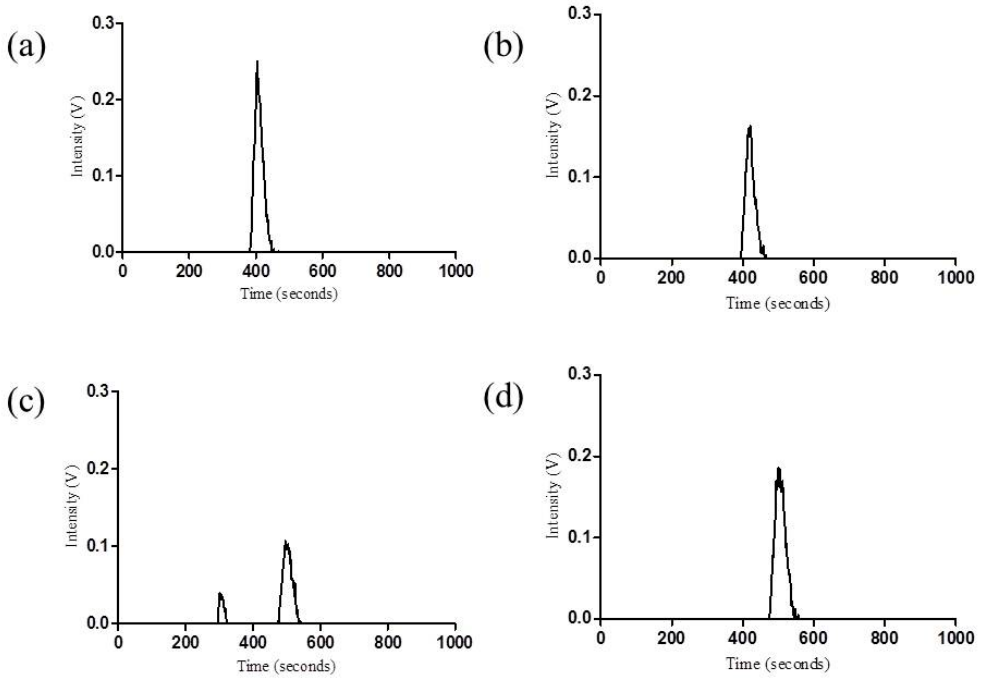


Figure 6.6: Partition HPLC chromatograms of supernatant samples obtained after incubation of radiolabeled lactose with fluidMAG-Amine immobilised (a) E537D β-Gal via EDC, (b) E537D β-Gal via glutaraldehyde, (c) wildtype β-Gal via EDC and (d) wildtype β-Gal via glutaraldehyde on a Sugar-Pak I column operated between 70 and 80 ^{o}C with 0.1 mM calcium EDTA as mobile phase.

6.3.4.3 Percent lactose binding

The percent lactose binding per immobilised E537D β-Gal sample was calculated and is summarised in Figure 6.7. It was calculated that the fluidMAG-

Amine immobilised E537D β-Gal via glutaraldehyde can bind and remove approximately 33 % of the lactose present in a 0.2 nmol solution while the EDC immobilised samples were only able to bind and remove approximately 9 % of the lactose present. These results obtained with radioactive partition HPLC, therefore, agree with the results from the samples analyzed by the scintillation counter, Figure 6.2. It is therefore evident that the glutaraldehyde immobilised E537D β-Gal can bind and remove significantly more lactose from solution than the EDC immobilized enzyme. These results confirmed the previous findings that glutaraldehyde is the preferred method for the functional immobilisation of β-Gal to fluidMAG-Amine.

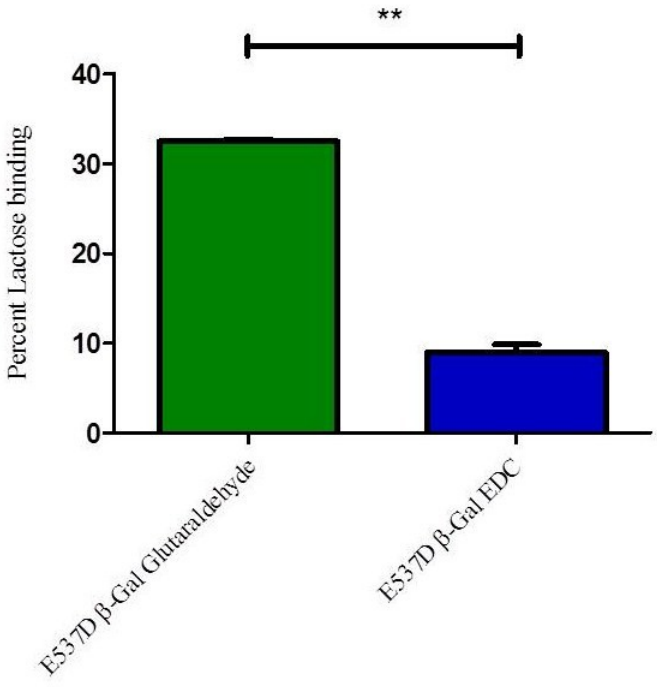


Figure 6.7: Comparison between percent lactose binding per immobilised E537D β-Gal sample via the two immobilisation strategies. The E537D β-Gal immobilised via glutaraldehyde was able to bind approximately 33 % of the lactose present while the E537D β-Gal immobilised via EDC was only able to bind approximately 9 % of the lactose present. Graphpad Prism 5 software was used for statistical analysis. Columns for the glutaraldehyde and EDC immobilised E537D β-Gal were compared by a t test (**P $<$ 0.05). Error bars indicate SEM, n = 2.

6.4 Conclusion

The retained activity of the fluidMAG-Amine immobilised E537D β-Gal via EDC and glutaraldehyde was investigated and, from the presented results, the glutaraldehyde strategy for covalent immobilisation of β-Gal was preferred. The glutaraldehyde strategy produced a more functionally immobilised enzyme, able to bind and remove more lactose from solution, when compared to the EDC strategy. The covalently immobilised E537D β-Gal was also able to remove twice (34 %) the amount of lactose as the physical adsorption method (15 %) (Figures 6.2 and 6.7) previously investigated in our laboratory [4].

Chapter 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Summary

Lactose free dairy products, or products with reduced lactose content, are in great demand world wide, since the majority of the world's population is lactose intolerant. This intolerance is as a result of a deficiency in the lactase enzyme produced in the small intestine. The large demand for lactose free products has encouraged researchers internationally to develop methods for the production of lactose free products on an industrial scale.

Current practice, for the production of lactose free products, include the hydrolysis of lactose to glucose and galactose by the addition of free, β-Gal or through so called bed reactors (refer to section 2.6.2). In the former method, it is custom to leave the added β-Gal enzyme in the milk after complete hydrolysis of the lactose as there is no facile technology available to remove the enzyme from the milk. The resulting lactose free product, produced by the addition of β-Gal, is excessively sweet due to the higher relative sweetness of glucose and galactose when compared to lactose. The excessive sweet taste is perceived as negative by the majority of consumers.

The lack of a practical and economical technology for the removal of lactose from milk, with the retention of the natural sweetness of the product, was the motivation for the development of a novel method for lactose removal from milk on an industrial scale.

7.1.1 β-Galactosidase

A number of sources for β-Gal have been indentified [9]. For the purpose of this study, *E. coli* was the source selected. The reasoning behind this selection was the fact that the genome of *E. coli* has been sequenced, it is a well characterized model organism for recombinant protein expression and most applications of this organism have already been optimized. Although *E. coli* does not have a generally recognized as safe (GRAS) status, it was an acceptable source for β-Gal since this study was still part of the proof of concept for the development of this novel technique to remove lactose from milk and not the final prototype. When considering the implementation of this novel technique in the food processing industry, attention must be given to the selection of an appropriate source of the enzyme. The appropriate source organism must have GRAS status, like *Saccharomyces*, *Lactobacillus acidophilus*, *L. bulgaricus*, *L. lactis*, *L. delbrueckii* and *Bacillus circulans* to ensure general acceptance by the public as well as compliance with current good manufacturing practices (GMP).

The *Lac Z* gene of *E. coli* was effectively cloned into the pTrcHis-TOPO plasmid and the E537D mutant was prepared as previously mentioned [4]. When expressed, these His-tagged constructs resulted in highly reproducible purification yields with IMAC (Figure 3.3). The results obtained after kinetic characterization of the pure wildtype and E537D β-Gal (Table 3.1) were similar to those previously achieved [4], with the mutant only retaining about 0.0146 times the activity of the wildtype while the substrate binding ability was not significantly altered.

7.1.2 Structural investigation of β-Gal

The three dimensional structure and topography investigation of the basic (Lys, Arg and His) and acidic (Asp and Glu) amino acid residues on the surface of β-Gal (Figure 4.6), as well as calculations regarding their abundance (Table 4.1), identified both the amine and carboxyl groups of β-Gal to be equally accessible for targeting during covalent coupling.

7.1.3 Covalent immobilisation of β-Gal via EDC and glutaraldehyde

The random covalent coupling of pure E537D β-Gal to fluidMAG-Amine was successfully achieved with both EDC and glutaraldehyde. EDC is a coupling agent that produces "zero-length" cross-linking, while glutaraldehyde is a cross-linking agent with a multi-component nature depending on the reaction conditions. Glutaraldehyde acts as a spacer when cross-linking protein and particles and ,therefore, limits structural hindrances. This covalent link between the protein and MNPs prevented enzyme leakage from the beads and added stability to the complex.

Optimization of the amount of protein coupled to fluidMAG-Amine, indicated that, when glutaraldehyde was used as the cross-linking agent, more protein could be coupled to the particles compared to the EDC method. The covalent coupling was verified and visualized experimentally via a range of verification methods including microscopy. It was apparent that gluaraldehyde was the more appropriate selection for the random covalent immobilisation of E537D β-Gal to fluidMAG-Amine, since it allowed for an increased carrier capacity of protein to the particles, which is possibly due to its multi-component nature [98].

7.1.4 Enzymatic activity of immobilised β-Gal

Initial analyses of the β-Gal-fluidMAG-Amine complex indicated non-specific protein binding to the nanoparticles at high protein concentrations (Figures 5.4 and 5.5). The non-specific binding was confirmed by SDS treatment of the complexes that removed non-covalently bound proteins from the particles, allowing a more accurate estimation of the covalent coupling of protein to the particles. As SDS is a harsh detergent, which lead to protein denaturation and inactivation, the detergents Tween-20 and -80 were investigated as alternative for the removal of non-specifically bound protein. These two detergents were less harsh than SDS for the desorption of non-covalently adsorbed protein and this was in agreement with observations made previously by Van den Berg *et al.* [95].

A comparison of the hydrolytic activity of the EDC and glutaraldehyde immobilised wildtype β-Gal, using ONPG as substrate, showed that the glutaraldehyde immobilised protein exhibited a two-fold higher activity than the EDC coupled protein (Figure 6.3), suggesting that, during glutaraldehyde cross-linking, the protein may be less prone to denaturation or conformational changes. EDC, on the other hand, appeared to influence these critical properties of β-Gal during coupling to fluidMAG-Amine. The activity of E537D β-Gal after immobilisation to fluidMAG-Amine was also investigated and it was found that this has definitely decreased but there were still enough activity left to ensure substrate binding and limited hydrolysis.

In accordance with the results obtained with the immobilised wildtype enzyme, the radioactive binding assay with radiolabelled lactose (D-glucose-1-^{14}C) demonstrated that the glutaraldehyde cross-linked E537D β-Gal-fluidMAG-Amine complex could physically bind and remove significantly more lactose than the EDC coupled complex. The glutaraldehyde cross-linked fluidMAG-E537D β-Gal was able to physically remove 34 % of the lactose from a 0.2 nmol/L lactose in solution. This was without any hydrolysis to the monosac-

charide sugars (glucose and galactose), while the glutaraldehyde cross-linked fluidMAG-wildtype β-Gal had, in the same incubation period, hydrolyzed all the lactose of the 0.2 nmol/L lactose solution (Figure 6.6). The 34 % lactose that the glutaraldehyde cross-linked fluidMAG-E537D β-Gal was able to remove, could be equated to approximately 0.07 nmol lactose bound per 1 mg fluidMAG-Amine containing an E537D β-Gal surface coating equal to 55 μg protein.

The immobilisation of a mutated form of β-Gal, on functionalized magnetic nanoparticles, was successful and could bind lactose and remove it from solution. The question that now needs to be addressed is the capacity of the complex to remove lactose from milk. When the lactose binding capacity, obtained in this study, is scaled up theoretically, it suggests that 1 kg of E537D β-Gal coated fluidMAG-Amine would be able to bind and remove approximately 0.070 mmol of lactose from solution. The lactose content of milk is in the order of 133 mmol/L and therefore suggests that over 900 incubation steps of 1 L milk with 1 kg nanoparticles would be necessary to reduce the lactose content by half.

In summary: The preceding discussion detailed the research objectives that were fulfilled experimentally. Specifically, it was shown that recombinant expressed and IMAC purified wildtype and E537D β-Gal could be covalently immobilised to commercially available fluidMAG-Amine through both EDC coupling and glutaraldehyde cross-linking. Glutaraldehyde was shown to be the preferred cross-linking agent for the MNP immobilisation of E537D β-Gal. It is apparent that this technology, at the current level of development, is not yet suited for industrial application. The idea itself is, however, novel and viable and a number of suggestions for future research and process optimization will be discussed.

Some of the methods and results of Dodd [4] were replicated during this study. These include wildtype and E537D β-Gal protein expression, IMAC

protein purification and β-Gal enzymatic activity determinations. However, Dodd made use of a physical adsorption, non-covalent, strategy (IMAC) to immobilise β-Gal to commercially available MNPs. This has shortcomings in that the link between the enzyme and solid support is not very strong and permanent, and might result in enzyme leakage. This investigation, however, made use of covalent cross-linking strategies to immobilise β-Gal to commercially available MNPs. This provides advantages, in that enzyme leakage was prevented. The new strategy incorporated was also able to bind and physically remove more lactose in comparison with Dodd's strategy [4].

7.2 Future research and recommendations

Solid supports, enzymes and immobilisation/coupling chemistries are rapidly expanding fields of study and therefore present a variety of opportunities for further improvements and applications. Due to the multi-disciplinary nature of this project, as well as the demand for the industrial production of lactose free products in the food process industry, the following areas for improvement are suggested.

7.2.1 MNPs

Commercially available MNPs were easy to obtain for the purpose of this study but expensive when considering the bulk production of protein coated particles. The synthesis of these magnetite nanoparticles is, however, not complicated or technically challenging. It would be more economical and practical, to develop tailor made particles for specific use in the dairy industry. The surface coating of the magnetite core, to functionalize the particles for covalent protein binding, is probably the more challenging component of MNP synthesis, but has progressed considerably at the Department of Chemistry and Polymer Science at the University of Stellenbosch where polymers for the facile non

covalent attachment of proteins have been developed [116].

7.2.2 The nanoparticle size and surface chemistry

The smaller the MNP size, the bigger the surface area to volume ratio and subsequently the capacity for the coupling of more protein per mg MNP. It is therefore important to synthesize MNPs with the smallest consistent size possible under controlled experimental conditions before applying surface chemistry to functionalize the particles for protein coupling.

The random covalent coupling of enzymes influences the substrate binding and catalytic abilities, as discussed in Chapter 6. Directed covalent anchoring of β-Gal to MNPs would limit the conformational changes and may even improve the accessibility of the active site for substrate binding, depending on the directed anchoring method applied. According to Teste *et al.* [58], the directed anchoring of biomolecules are more reproducible than random methods. The directed covalent anchoring of biomolecules also allow for coupling in an orderly fashion as well as the use of linkers or spacers to limit conformational changes and steric hindrances [61].

A directed covalent anchoring method that could be attempted in the future is described by Pessela *et al.* [59]. These authors described the use of "novel heterofunctional chelate-epoxy sepabeads". The surface chemistry of these beads consisted of a low concentration of metal chelated groups and a high density of epoxy groups [59]. These heterofunctional beads allowed for the selective adsorption of poly-His-tagged proteins through IMAC chemistry to the chelated groups whereafter the selective covalent coupling was introduced through interactions with the epoxy groups [59].

7.2.3 Lactose binding protein - size considerations

The large size (464 kDa) of β-Gal limits the amount of enzyme that can be immobilised to the surface of MNPs and ultimately the amount of lactose

bound and removed. Considering the use of another enzyme, able to bind lactose, with a significantly smaller molecular mass would potentially allow for more enzyme to be coupled to per mg of MNPs, and subsequently increase the ability to bind and remove more lactose from solution. Although there is currently no other enzyme known to be able to bind and hydrolyze lactose, a number of disaccharide binding enzymes exist, which could be investigated and mutated to obtain lactose binding.

In conclusion: The investigation into and implementation of some or all of the above mentioned recommendations for the optimization of this novel technique could result in the development of technology that would allow for the removal of lactose from milk and the production of lactose free dairy products, or products with reduced lactose content, on an industrial scale.

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